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<b>(54) Title:</b> ADENOVIRUS DERIVED GENE DELIVERY VEHICLES COMPRISING AT LEAST ONE ELEMENT OF ADENOVIRUS TYPE 35  <b>(57) Abstract</b> <p>The serotypes differ in their natural tropism. The adenovirus serotypes (2, 4, 5 and 7) all have a natural affiliation towards lung epithelia and other respiratory tissues. In contrast, serotypes (40 and 41) have a natural affiliation towards the gastrointestinal tract. The serotypes described above, differ in at least capsid proteins (penton-base, hexon), proteins responsible for cell binding (fiber protein), and proteins involved in adenovirus replication. This difference in tropism and capsid protein among serotypes has led to the many research efforts aimed at redirecting the adenovirus tropism by modification of the capsid proteins.</p>		

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ADENOVIRUS DERIVED GENE DELIVERY VEHICLES COMPRISING  
AT LEAST ONE ELEMENT OF ADENOVIRUS TYPE 35

The present invention relates to the field of gene therapy, in particular gene therapy involving elements derived from viruses, more in particular elements of adenoviruses.

Adenoviruses have been proposed as suitable vehicles to  
5 deliver genes to the host.

There are a number of features of adenoviruses that make them particularly useful for the development of gene-transfer vectors for human gene therapy:

The adenovirus genome is well characterized. It consists of  
10 a linear double-stranded DNA molecule of approximately 36000 base pairs. The adenovirus DNA contains identical Inverted Terminal Repeats (ITR) of approximately 90-140 base pairs with the exact length depending on the serotype. The viral origins of replication are within the ITRs exactly at the  
15 genome ends;

The biology of the adenoviruses is characterized in detail; the adenovirus is not associated with severe human pathology in immuno-competent individuals

The virus is extremely efficient in introducing its DNA into  
20 the host cell; the virus can infect a wide variety of cells and has a broad host-range;

The virus can be produced at high virus titers in large quantities;

The virus can be rendered replication defective by deletion  
25 of the early-region 1 (E1) of the viral genome (Brody et al, 1994). Most adenoviral vectors currently used in gene therapy have a deletion in the E1 region, where desired genetic information can be introduced.

30 Based on these features, preferred methods for in vivo gene transfer into human target cells, make use of adenoviral vectors as gene delivery vehicles.

However, there are still drawbacks associated with the therapeutic use of adenoviral vectors in humans. A major

drawback is the existence of widespread pre-existing immunity among the population against adenoviruses. Exposure to wild-type adenoviruses is very common in humans, as has been documented extensively [reviewed in Wadell, 1984]. This exposure has resulted in immune responses against most types of adenoviruses, not alone against adenoviruses to which individuals have actually been exposed, but also against adenoviruses which have similar (neutralizing) epitopes. This phenomenon of pre-existing antibodies in humans, in combination with a strong secondary humoral and cellular immune response against the virus, can seriously affect gene transfer using recombinant adenoviral vectors. To date, six different subgroups of human adenoviruses have been proposed which in total encompasses 51 distinct adenovirus serotypes (see table 1). A serotype is defined on the basis of its immunological distinctiveness as determined by quantitative neutralization with animal antisera (horse, rabbit). If neutralization shows a certain degree of cross-reaction between two viruses, distinctiveness of serotype is assumed if A) the hemagglutinins are unrelated, as shown by lack of cross-reaction on hemagglutination-inhibition, or B) substantial biophysical/ biochemical differences in DNA exist (Francki et al, 1991). The nine serotypes identified last (42-51) were isolated for the first time from HIV-infected patients (Hierholzer et al 1988; Schnurr et al 1993;). For reasons not well understood, most of such immune-compromised patients shed adenoviruses that were rarely or never isolated from immune-competent individuals (Hierholzer et al 1988, 1992; Khoo et al, 1995, De Jong et al, 1998).

The vast majority of individuals have had previous exposure to adenoviruses, especially the well investigated adenovirus serotypes 5 and type 2 (Ad5 and Ad2) or immunologically related serotypes. Importantly, these two serotypes are also the most extensively studied for use in human gene therapy.

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from other (adeno) viruses, as long as one replaces an element which could lead to immunity against such a gene delivery vehicle by an element of adenovirus 35 or a functional homologue thereof, which has less of such a drawback and which preferably avoids such a drawback. In the present invention a gene delivery vehicle is any vehicle that is capable of delivering a nucleic acid of interest to a host cell. It must, according to the invention comprise an element of adenovirus 35 or a functional equivalent of such an element, which must have a beneficial effect regarding the immune response against such a vehicle. Basically all other elements making up the vehicle can be any elements known in the art or developed in the art, as long as together they are capable of delivering said nucleic acid of interest. In principle the person skilled in the art can use and/or produce any adenoviral products or production systems that can or have been applied in the adenoviral field. Typically the products of the invention can be made in the packaging cells useable for e.g. adenovirus 5, typically the vectors based on adenovirus 35 can be produced and/or used in the same manner as those of other adenoviruses e.g. adenovirus 2 and/or 5. A good overview of the possibilities of minimal vectors, packaging systems, intracellular amplification, vector and plasmid based systems can be found in applicant's copending applications (PCT/NL99/00235 and PCT/NL96/00244) incorporated herein by reference. Non-viral delivery systems can also be provided with elements according to the invention as can viral delivery systems. Both kinds of systems are well known in the art in many different set-ups and do therefor not need any further elaboration here. A review on the many different systems and their properties can be found in Robbins and Ghivizzani (1998) and in Prince (1998) incorporated herein by reference.

Gene delivery vehicles typically contain a nucleic acid of interest. A nucleic acid of interest can be a gene or a

- functional part of a gene (wherein a gene is any nucleic acid which can be expressed) or a precursor of a gene or a transcribed gene on any nucleic acid level (DNA and/or RNA: double or single stranded). Genes of interest are well known in the art and typically include those encoding therapeutic proteins such as TPA, EPO, cytokines, antibodies or derivatives thereof, etc. An overview of therapeutic proteins to be applied in gene therapy are listed below.
- 10 Immune-stimulatory factors like tumor-specific antigens, cytokines, etc.;
- Anti-angiogenic factors non-limiting examples endostatin, angiostatin, ATF-BPTI CDT-6, dominant negative VEGF-mutants, etc.;
- 15 Angiogenic factors non-limiting example VEGF; Fibroblast growth factors, Nitric oxide synthases, C-type natriuretic peptide, etc.;
- Inflammation inhibiting proteins like soluble CD40, FasL, IL-12, IL-10, IL-4, IL-13 and excreted single chain
- 20 antibodies to CD4, CD5, CD7, CD52, IL-2, IL-1, IL-6, TNF, etc. or excreted single chain antibodies to the T-cell receptor on the auto-reactive T-cells. Also, dominant negative mutants of PML may be used to inhibit the immune response.
- 25 Furthermore, antagonists of inflammation promoting cytokines may be used, for example IL-1RA(receptor antagonist) and soluble receptors like sIL-1RI, sIL-1RII, sTNFR1 and sTNFR2. Growth and/or immune response inhibiting genes such as ceNOS, Bcl3, cactus and I $\kappa$ B $\alpha$ ,  $\beta$  or  $\gamma$  and apoptosis
- 30 inducing proteins like the VP3 protein of chicken anemia virus may also be used. Furthermore, suicide genes like HSV-TK, cytosine deaminase, nitroreductase and linamerase may be used.
- 35 A nucleic acid of interest may also be a nucleic acid which can hybridise with a nucleic acid sequence present in the

host cell thereby inhibiting expression or transcription or translation of said nucleic acid. It may also block through cosuppression. In short a nucleic acid of interest is any nucleic acid that one may wish to provide a cell with in order to induce a response by that cell, which response may be production of a protein, inhibition of such production, apoptosis, necrosis, proliferation, differentiation etc. The present invention is the first to disclose adenovirus 35 or a functional homologue thereof for therapeutical use, therefor the invention also provides an adenovirus serotype 35 or a functional homologue thereof or a chimaeric virus derived therefrom, or a gene delivery vehicle based on said virus its homologue or its chimaera for use as a pharmaceutical. The serotype of the present invention, adenovirus type 35, is in itself known in the art. It is an uncommon group B adenovirus that was isolated from patients with acquired immunodeficiency syndrome and other immunodeficiency disorders (Flomenberg et al., 1987; De Jong et al., 1983). Ad 35 has been shown to differ from the more fully characterized subgroup C (including Ad2 and Ad5) with respect to pathogenic properties (Basler et al., 1996). It has been suggested that this difference may be correlated with differences in the E3 region of the Ad35 genome (Basler et al., 1996). The DNA of Ad35 has been partially cloned and mapped (Kang et al., 1989a and b; Valderrama-Leon et al., 1985).

B type adenovirus serotypes such as 34 and 35 have a different E3 region than other serotypes. Typically this region is involved in suppressing immune response to adenoviral products. Thus the invention provides a gene delivery vehicle according to the invention whereby said elements involved in avoiding or diminishing immune response comprise adenovirus 35 E3 expression products or the genes encoding them or functional equivalents of either or both.



Another part of adenoviruses involved in immune responses is the capsid, in particular the penton and/or the hexon proteins. Thus the invention also provides a gene delivery vehicle according to the invention whereby the elements  
5 comprise at least one adenovirus 35 capsid protein or functional part thereof, such as fiber, penton and/or hexon proteins or a gene encoding at least one of them. It is not necessary that a whole protein relevant for immune response is of adenovirus 35 (or a functional homologue thereof)  
10 origin. It is very well possible to insert a part of an adenovirus fiber, penton or hexon protein into another fiber, penton or hexon. Thus chimaeric proteins are obtained.

It is also possible to have a penton of a certain  
15 adenovirus, a hexon from another and a fiber or an E3 region from yet another adenovirus. According to the invention at least one of the proteins or genes encoding them should comprise an element from adenovirus 35 or a functional homologue thereof, whereby said element has an effect on the  
20 immune response of the host. Thus the invention provides a gene delivery according to the invention, which is a chimaera of adenovirus 35 with at least one other adenovirus. In this way one can also modify the resulting virus in other aspects than the immune response alone. One  
25 can enhance its efficiency of infection with elements responsible therefor; one can enhance its replication on a packaging cell, or one can change its tropism.

Thus the invention e.g. provides a gene delivery vehicle according to the invention which has a different tropism  
30 than adenovirus 35. Of course the tropism should be altered preferably such that the gene delivery vehicle is delivered preferentially to a subset of the host's cells, i.e. the target cells. Changes in tropism and other changes which can also be applied in the present invention of adenoviral or  
35 other gene delivery vehicles are disclosed in applicant's copending applications (nos. 98204482.8, 99200624.7 and

98202297.2) incorporated herein by reference. Of course the present application also provides any and all building blocks necessary and/or useful to get to the gene delivery vehicles and/or the chimaeras, etc. of the present invention. This includes packaging cells such as PER.C6 (ECACC deposit number 96022940) or cells based thereon, but adapted for Ad35 or a functional homologue thereof; it also includes any nucleic acids encoding functional parts of adenovirus 35 or a functional homologue thereof, such as helper constructs and packaging constructs, as well as vectors comprising genes of interest and e.g. an ITR, etc. Typically applicant's application (PCT/NL96/00244) incorporated herein by reference, discloses elements necessary and useful for arriving at the invented gene delivery vehicles. Thus the invention also provides a nucleic acid encoding at least a functional part of a gene delivery vehicle according to the invention, or a virus, homologue or chimaera thereof according to the invention. According to the invention, such elements, which encode functions that will end up in the resulting gene delivery vehicle must comprise or be encoded by a nucleic acid encoding at least one of the adenovirus serotype 35 elements or a functional equivalent thereof, responsible for avoiding or diminishing neutralising activity against adenoviral elements by the host to which the gene is to be delivered. Typically the gene of interest would be present on the same nucleic acid which means that such a nucleic acid has such a gene or that it has a site for introducing a gene of interest therein.

Typically such a nucleic acid also comprises at least one ITR and if it is a nucleic acid to be packaged also a packaging signal. However, as mentioned before all necessary and useful elements and/or building blocks for the present invention can be found in applicant's application (PCT/NL96/00244). A set of further improvements in the field of producing adenoviral gene delivery vehicles is

applicant's plasmid system disclosed in PCT/NL99/00235 mentioned herein before. This system works in one embodiment as a homologous recombination of an adapter plasmid and a longer plasmid, together comprising all elements of the nucleic acid to be incorporated in the gene delivery vehicle. These methods can also be applied to the presently invented gene delivery vehicles and their building elements. Thus the invention also provides a nucleic acid according to the invention further comprising a region of nucleotides designed or useable for homologous recombination, preferably as part of at least one set of two nucleic acids comprising a nucleic acid according to the invention, whereby said set of nucleic acids is capable of a single homologous recombination event with each other, which leads to a nucleic acid encoding a functional gene delivery vehicle. Both empty packaging cells (in which the vector to be packaged to make a gene delivery vehicle according to the invention still has to be introduced or produced) as well as cells comprising a vector according to the invention to be packaged are provided. Thus the invention also encompasses a cell comprising a nucleic acid according to the invention or a set of nucleic acids according to the invention, preferably a cell which complements the necessary elements for adenoviral replication which are absent from the nucleic acid according to be packaged, or from a set of nucleic acids according to the invention. In the present invention it has been found that E1-deleted adenovirus 35 vectors, are not capable of replication on cells that provide adenovirus 5 proteins *in trans*. The invention therefore further provides a cell capable of providing adenovirus 35 E1 proteins *in trans*. Such a cell is typically a human cell derived from the retina or the kidney. Embryonal cells such as amniocytes, have been shown to be particularly suited for the generation of an E1 complementing cell line. Such cells are therefor preferred in the present invention. Serotype specific complementation by E1 proteins can be due to one or

more protein(s) encoded by the E1 region. It is therefor essential that at least the serotype specific protein is provided in trans in the complementing cell line. The non-serotype specific E1 proteins essential for effective

5 complementation of an E1-deleted adenovirus can be derived from other adenovirus serotypes. Preferably, at least an E1 protein from the E1B region of adenovirus 35 is provided in trans to complement E1-deleted adenovirus 35 based vectors. In one embodiment nucleic acid encoding the one or more

10 serotype specific E1-proteins is introduced into the PER.C6 cell or a cell originating from a PER.C6 cell (ECACC deposit number 96022940), or a similar packaging cell complementing with elements from Ad 35 or a functional homologue thereof. As already alluded to the invention also encompasses a

15 method for producing a gene delivery vehicle according to the invention, comprising expressing a nucleic acid according to the invention in a cell according to the invention and harvesting the resulting gene delivery vehicle. The above refers to the filling of the empty

20 packaging cell with the relevant nucleic acids. The format of the filled cell is of course also part of the present invention, which provides a method for producing a gene delivery vehicle according to the invention, comprising culturing a filled packaging cell (producer cell) according

25 to the invention in a suitable culture medium and harvesting the resulting gene delivery vehicle. The resulting gene delivery vehicles obtainable by any method according to the invention are of course also part of the present invention, particularly also a gene delivery

30 vehicle according to the invention, which is derived from a chimaera of an adenovirus and an integrating virus. It is well known that adenoviral gene delivery vehicles do not integrate into the host genome normally. For long term expression of genes in a host cell it is therefor preferred

35 to prepare chimaeras which do have that capability. Such chimaeras have been disclosed in our copending application

PCT/NL98/00731 incorporated herein by reference. A very good example of such a chimaera of an adenovirus and an integrating virus wherein said integrating virus is an adeno associated virus. As discussed hereinbefore other useful chimaeras, which can also be combined with the above are chimaeras (be it in swapping whole proteins or parts thereof or both) which have altered tropism. A very good example thereof is a chimaera of Ad 35 and Ad 16, possibly with elements from for instance Ad 2 or Ad 5, wherein the tropism determining part of Ad 16 or a functional equivalent thereof is used to direct the gene delivery vehicle to synoviocytes and/or smooth muscle cells (see our copending applications nos. 98204482.8 and 99200624.7) incorporated herein by reference). Dendritic cells (DC) and hemopoietic stem cells (HSC) are not easily transduced with Ad2 or Ad5 derived gene delivery vehicles. The present invention provides gene delivery vehicles that possess increased transduction capacity of DC and HSC cells. Such gene delivery vehicles at least comprises the tissue tropism determining part of an Ad35 adenovirus. The invention therefore further provides the use of a tissue tropism determining part of an adenovirus 35 capsid for transducing dendritic cells and/or hemopoietic stem cells. Other B-type adenoviruses are also suited. A tissue tropism determining part comprises at least the knob and/or the shaft of a fiber protein. Of course it is very well possible for a person skilled in the art to determine the amino acid sequences responsible for the tissue tropism in the fiber protein. Such knowledge can be used to devise chimeric proteins comprising such amino acid sequences. Such chimeric proteins are therefor also part of the invention. DC cells are very efficient antigen presenting cells. By introducing the gene delivery vehicle into such cells the immune system of the host can be triggered to toward specific antigens. Such antigens can be encoded by nucleic acid delivered to the DC or by the proteins of the gene delivery vehicle it self. The present

invention therefor also provides a gene delivery vehicle with the capacity to evade to host immune system as a vaccine. The vector being capable to evade the immune system long enough to efficiently find it target cells and at the same time capable of delivering specific antigens to antigen presenting cells thereby allowing the induction and/or stimulation of an efficient immune responses toward the specific antigen(s). To further modulate the immune response, the gene delivery vehicle may comprise proteins and/or nucleic encoding such proteins capable of modulating an immune response. Non-limiting examples of such proteins are found among the interleukins, the adhesion molecules, the co-stimulatory proteins, the interferons etc. The invention therefore further provides a vaccine comprising a gene delivery vehicle of the invention. The invention further provides an adenovirus vector with the capacity to efficiently transduce DC and/or HSC, the vehicle comprising at least a tissue tropism determining part of serotype 35 adenvirus. The invention further provides the use of such delivery vehicles for the transduction of HSC and/or DC cells. Similar tissue tropisms are found among other adenoviruses of serotype B, particularly in serotype 11 and are also part of the invention. Of course it is also possible to provide other gene delivery vehicles with the tissue tropism determining part thereby providing such delivery vehicles with an enhanced DC and/or HSC transduction capacity. Such gene delivery vehicles are therefor also part of the invention.

The gene delivery vehicles according to the invention can be used to deliver genes or nucleic acids of interest to host cells. This will typically be a pharmaceutical use. Such a use is included in the present invention. Compositions suitable for such a use are also part of the present invention. The amount of gene delivery vehicle that needs to be present per dose or per infection (m.o.i) will depend on the condition to be treated, the route of administration

(typically parenteral) the subject and the efficiency of infection, etc. Dose finding studies are well known in the art and those already performed with other (adenoviral) gene delivery vehicles can typically be used as guides to find  
5 suitable doses of the gene delivery vehicles according to the invention. Typically this is also where one can find suitable excipients, suitable means of administration, suitable means of preventing infection with the vehicle where it is not desired, etc. Thus the invention also  
10 provides a pharmaceutical formulation comprising a gene delivery vehicle according to the invention and a suitable excipient, as well as a pharmaceutical formulation comprising an adenovirus, a chimaera thereof, or a functional homologue thereof according to the invention and  
15 a suitable excipient.

#### Detailed description

As described above, the most extensively studied serotypes  
20 of adenovirus are not ideally suitable for delivering additional genetic material to host cells. This is partly due to the pre-existing immunity among the population against these serotypes. This presence of pre-existing antibodies in humans, in combination with a strong secondary  
25 humoral and cellular immune response against the virus will affect adenoviral gene therapy.

The present invention provides the use of at least elements of a serotype and functional homologues thereof of  
30 adenovirus which are very suitable as gene therapy vectors. The present invention also discloses an automated high-throughput screening of all known adenovirus serotypes against sera from many individuals. Surprisingly, no neutralizing ability was found in any of the sera that were  
35 evaluated against one particular serotype, adenovirus 35 (Ad35). This makes the serotype of the present invention

extremely useful as a vector system for gene therapy in man. Such vector system is capable of efficiently transferring genetic material to a human cell without the inherent problem of pre-existing immunity.

5 Typically, a virus is produced using an adenoviral vector (typically a plasmid, a cosmid or baculovirus vector). Such vectors are of course also part of the present invention. The invention also provides adenovirus derived vectors that have been rendered replication defective by deletion or  
10 inactivation of the E1 region. Of course, also a gene of interest can be inserted at for instance the site of E1 of the original adenovirus from which the vector is derived. In all aspects of the invention the adenoviruses may contain deletions in the E1 region and insertions of heterologous  
15 genes linked either or not to a promoter. Furthermore, the adenoviruses may contain deletions in the E2, E3 or E4 regions and insertions of heterologous genes linked to a promoter. In these cases, E2 and/or E4 complementing cell lines are required to generate recombinant adenoviruses.

20 One may choose to use the Ad35 serotype itself for the preparation of recombinant adenoviruses to be used in gene therapy. Alternatively, one may choose to use elements derived from the serotype of the present invention in such  
25 recombinant adenoviruses. One may for instance develop a chimaeric adenovirus that combines desirable properties from different serotypes. Some serotypes have a somewhat limited host range, but have the benefit of being less immunogenic, some are the other way round. Some have a problem of being  
30 of a limited virulence, but have a broad host range and/or a reduced immunogenicity. Such chimaeric adenoviruses are known in the art, and they are intended to be within the scope of the present invention. Thus in one embodiment the invention provides a chimaeric adenovirus comprising at  
35 least a part of the adenovirus genome of the present serotype, providing it with absence of pre-existing



immunity, and at least a part of the adenovirus genome from another adenovirus serotype resulting in a chimaeric adenovirus. In this manner the chimaeric adenovirus produced is such that it combines the absence of pre-existing  
5 immunity of the serotype of the present invention, to other characteristics of another serotype. Such characteristics may be temperature stability, assembly, anchoring, redirected infection, production yield, redirected or improved infection, stability of the DNA in the target cell,  
10 etc.

A packaging cell will generally be needed in order to produce sufficient amount of adenoviruses. For the production of recombinant adenoviruses for gene therapy purposes, several cell lines are available. These include  
15 but are not limited to the known cell lines PER.C6 (ECACC deposit number 96022940), 911, 293, and El A549.

An important feature of the present invention is the means to produce the adenovirus. Typically, one does not want an adenovirus batch for clinical applications to contain  
20 replication competent adenovirus. In general therefore, it is desired to omit a number of genes (but at least one) from the adenoviral genome on the adenoviral vector and to supply these genes in the genome of the cell in which the vector is brought to produce chimaeric adenovirus. Such a cell is  
25 usually called a packaging cell. The invention thus also provides a packaging cell for producing an adenovirus (a gene delivery vehicle) according to the invention, comprising in trans all elements necessary for adenovirus production not present on the adenoviral vector according to  
30 the invention. Typically vector and packaging cell have to be adapted to one another in that they have all the necessary elements, but that they do not have overlapping elements which lead to replication competent virus by recombination.

35

Thus the invention also provides a kit of parts comprising a packaging cell according to the invention and a recombinant vector according to the invention whereby there is essentially no sequence overlap leading to recombination resulting in the production of replication competent adenovirus between said cell and said vector.

Thus the invention provides methods for producing adenovirus, which upon application will escape pre-existing humoral immunity, comprising providing a vector with elements derived from an adenovirus serotype against which virtually no natural immunity exists and transfecting said vector in a packaging cell according to the invention and allowing for production of viral particles.

In one aspect this invention describes the use of the adenovirus serotype or the present invention to overcome natural existing or induced, neutralising host activity towards adenoviruses administered in vivo for therapeutic applications. The need for a new serotype is stressed by observations that 1) repeated systemic delivery of recombinant adenovirus serotype 5 is unsuccessful due to formation of high titers of neutralising antibodies against the recombinant adenovirus serotype 5 (Schulick et al, 1997), and 2) pre-existing or humoral immunity is widespread in the population.

In another aspect this invention provides the use of gene delivery vehicles of the invention or the use of adenovirus serotype 35 for vaccination purposes. Such use prevents at least in part undesired immune responses of the host. Non-limiting examples of undesired immune responses are evoking an immune response against the gene delivery vehicle or adenovirus serotype 35 and/or boosting of an immune response against the gene delivery vehicle or adenovirus serotype 35. In another aspect of the invention, alternating use is made of Ad vectors belonging to different subgroups. This aspect

of the invention therefore circumvents the inability to repeat the administration of an adenovirus for gene therapy purposes.

## Example 1

A high throughput assay for the detection of neutralising  
activity in human serum

5

To enable screening of a large amount of human sera for the presence of neutralising antibodies against all adenovirus serotypes, an automated 96-wells assay was developed.

*Human sera*

10

A panel of 100 individuals was selected. Volunteers (50% male, 50% female) were healthy individuals between 20 and 60 years old with no restriction for race. All volunteers signed an informed consent form. People professionally involved in adenovirus research were

15

excluded.

Approximately 60 ml blood was drawn in dry tubes. Within two hours after sampling, the blood was centrifuged at 2500 rpm for 10 minutes. Approximately 30 ml serum was transferred to polypropylene tubes and stored frozen at -20°C until further

20

use.

Serum was thawed and heat-inactivated at 56°C for 10 minutes and then aliquotted to prevent repeated cycles of freeze/thawing. Part was used to make five steps of twofold dilutions in medium (DMEM, Gibco BRL) in a quantity enough to fill out approximately 70 96-well plates. Aliquots of undiluted and diluted sera were pipetted in deep well plates (96-well format) and using a programmed platemate dispensed in 100 µl aliquots into 96-well plates. This way the plates were loaded with eight different sera in duplo (100 µl/well)

25

30 according to the scheme below:

S1/ 2	S1/ 4	S1/ 8	S1/ 16	S1/ 32	S5/ 2	S5/ 4	S5/ 8	S5/ 16	S5/ 32	-	-
S1/ 2	S1/ 4	S1/ 8	S1/ 16	S1/ 32	S5/ 2	S5/ 4	S5/ 8	S5/ 16	S5/ 32	-	-
S2/ 2	S2/ 4	S2/ 8	S2/ 16	S2/ 32	S6/ 2	S6/ 4	S6/ 8	S6/ 16	S6/ 32	-	-
S2/ 2	S2/ 4	S2/ 8	S2/ 16	S2/ 32	S6/ 2	S6/ 4	S6/ 8	S6/ 16	S6/ 32	-	-
S3/ 2	S3/ 4	S3/ 8	S3/ 16	S3/ 32	S7/ 2	S7/ 4	S7/ 8	S7/ 16	S7/ 32	-	-
S3/ 2	S3/ 4	S3/ 8	S3/ 16	S3/ 32	S7/ 2	S7/ 4	S7/ 8	S7/ 16	S7/ 32	-	-
S4/ 2	S4/ 4	S3/ 8	S3/ 16	S3/ 32	S8/ 2	S8/ 4	S8/ 8	S8/ 16	S8/ 32	-	-
S4/ 2	S4/ 4	S3/ 8	S3/ 16	S3/ 32	S8/ 2	S8/ 4	S8/ 8	S8/ 16	S8/ 32	-	-

Where S1/2 to S8/2 in columns 1 and 6 represent 1x diluted sera and Sx/4, Sx/8, Sx/16 and Sx/32 the twofold serial dilutions. The last plates also contained four wells filled with 100  $\mu$ l foetal calf serum as a negative control. Plates were kept at -20°C until further use.

#### *Preparation of human adenovirus stocks*

10 Prototypes of all known human adenoviruses were inoculated on T25 flasks seeded with PER.C6 cells (ECACC deposit number 96022940) (Fallaux et al., 1998) and harvested upon full CPE. After freeze/thawing 1-2 ml of the crude lysates was used to inoculate a T80 flask with PER.C6 cells (ECACC deposit number 96022940) and virus was harvested at full CPE. The timeframe between inoculation and occurrence of CPE as well as the amount of virus needed to re-infect a new culture, differed between serotypes. Adenovirus stocks were prepared by freeze/thawing and used to inoculate 3-4 T175 cm<sup>2</sup> three-layer flasks with PER.C6 cells (ECACC deposit number 96022940). Upon occurrence of CPE, cells were harvested by tapping the flask, pelleted and virus was isolated and purified by a two step CsCl gradient as follows. Cell pellets were dissolved in 50 ml 10 mM NaPO<sub>4</sub> buffer (pH 7.2) and frozen at -20°C. After thawing at 37°C,

5.6 ml sodium deoxycholate (5% w/v) was added. The solution was mixed gently and incubated for 5-15 minutes at 37°C to completely lyse the cells. After homogenizing the solution, 1875  $\mu$ l 1M MgCl<sub>2</sub> was added. After the addition of 375  $\mu$ l DNase (10 mg/ml) the solution was incubated for 30 minutes at 37°C. Cell debris was removed by centrifugation at 1880xg for 30 minutes at RT without brake. The supernatant was subsequently purified from proteins by extraction with freon (3x). The cleared supernatant was loaded on a 1M Tris/HCl buffered cesiumchloride blockgradient (range: 1.2/1.4 gr/ml) and centrifugated at 21000 rpm for 2.5 hours at 10°C. The virus band is isolated after which a second purification using a 1M Tris/HCl buffered continuous gradient of 1.33 gr/ml of cesiumchloride was performed. The virus was then centrifuged for 17 hours at 55000 rpm at 10°C. The virus band is isolated and sucrose (50 % w/v) is added to a final concentration of 1%. Excess cesiumchloride is removed by dialysis (three times 1 hr at RT) in dialysis slides (Slide-a-lizer, cut off 10000 kDa, Pierce, USA) against 1.5 ltr PBS supplemented with CaCl<sub>2</sub> (0.9 mM), MgCl<sub>2</sub> (0.5mM) and an increasing concentration of sucrose (1, 2, 5%). After dialysis, the virus is removed from the slide-a-lizer after which it is aliquoted in portions of 25 and 100  $\mu$ l upon which the virus is stored at -85°C.

To determine the number of virus particles per milliliter, 50  $\mu$ l of the virus batch is run on a high-pressure liquid chromatograph (HPLC) as described by Shabram et al (1997). Viruses were eluted using an NaCl gradient ranging from 0 to 600 mM. As depicted in table I, the NaCl concentration by which the viruses were eluted differed significantly among serotypes.

Most human adenoviruses replicated well on PER.C6 cells ((ECACC deposit number 96022940) with a few exceptions. Adenovirus type 8 and 40 were grown on 911-E4 cells (He et al., 1998). Purified stocks contained between  $5 \times 10^{10}$  and  $5 \times 10^{12}$  virus particles/ml (VP/ml; see table I).

*Titration of purified human adenovirus stocks*

Adenoviruses were titrated on PER.C6 cells (ECACC deposit number 96022940) to determine the amount of virus necessary to obtain full CPE in five days, the length of the neutralisation assay. Hereto, 100  $\mu$ l medium was dispensed into each well of 96-well plates. 25  $\mu$ l of adenovirus stocks prediluted  $10^4$ ,  $10^5$ ,  $10^6$  or  $10^7$  times were added to column 2 of a 96-well plate and mixed by pipetting up and down 10 times. Then 25  $\mu$ l was brought from column 2 to column 3 and again mixed. This was repeated until column 11 after which 25  $\mu$ l from column 11 was discarded. This way serial dilutions in steps of 5 were obtained starting off from a prediluted stock. Then  $3 \times 10^4$  PER.C6 cells (ECACC deposit number 96022940) were added in a 100  $\mu$ l volume and the plates were incubated at 37 °C, 5% CO<sub>2</sub> for five or six days. CPE was monitored microscopically. The method of Reed and Muensch was used to calculate the cell culture inhibiting dose 50% (CCID50).

In parallel identical plates were set up that were analysed using the MTT assay (Promega). In this assay living cells are quantified by colorimetric staining. Hereto, 20  $\mu$ l MTT (7.5 mgr/ml in PBS) was added to the wells and incubated at 37 °C, 5% CO<sub>2</sub> for two hours. The supernatant was removed and 100  $\mu$ l of a 20:1 isopropanol/triton-X100 solution was added to the wells. The plates were put on a 96-wells shaker for 3-5 minutes to solubilise precipitated staining. Absorbance was measured at 540 nm and at 690 nm (background). By this assay wells with proceeding CPE or full CPE can be distinguished.

*Neutralisation assay*

96-well plates with diluted human serum samples were thawed at 37 °C, 5% CO<sub>2</sub>. Adenovirus stocks diluted to 200 CCID50 per 50  $\mu$ l were prepared and 50  $\mu$ l aliquots were added to columns 1-11 of the plates with serum. Plates were incubated

for 1 hour at 37°C, 5% CO<sub>2</sub>. Then 50 µl PER.C6 cells (ECACC deposit number 96022940) at 6x10<sup>5</sup>/ml were dispensed in all wells and incubated for 1 day at 37 °C, 5% CO<sub>2</sub>. Supernatant was removed using fresh pipet tips for each row and 200 µl fresh medium was added to all wells to avoid toxic effects of the serum. Plates were incubated for another 4 days at 37 °C, 5% CO<sub>2</sub>. In addition, parallel control plates were set up in duplo with diluted positive control sera generated in rabbits and specific for each serotype to be tested in rows A and B and with negative control serum (FCS) in rows C and D. Also, in each of the rows E-H a titration was performed as described above with steps of five times dilutions starting with 200 CCID<sub>50</sub> of each virus to be tested. On day 5 one of the control plates was analysed microscopically and with the MTT assay. The experimental titer was calculated from the control titration plate observed microscopically. If CPE was found to be complete, i.e. the first dilution in the control titration experiment analysed by MTT shows clear cell death, all assay plates were processed. If not, the assay was allowed to proceed for one or more days until full CPE was apparent after which all plates were processed. In most cases the assay was terminated at day 5. For Ad1, 5, 33, 39, 42 and 43 the assay was left for six days and for Ad2 for eight days.

A serum sample is regarded to be non-neutralising when at the highest serum concentration a maximum protection is seen of 40% compared to the controls without serum.

The results of the analysis of 44 prototype adenoviruses against serum from 100 healthy volunteers is shown in figure 1. As expected the percentage of serum samples that contained neutralising antibodies to Ad2 and Ad5 was very high. This was also true for most of the lower numbered adenoviruses. Surprisingly, none of the serum samples contained neutralising antibodies to adenovirus serotype 35.

Also, the number of individuals with neutralising antibody titers to the serotypes 26, 34 and 48 was very low.



Therefor, recombinant E1-deleted adenoviruses based on Ad35 or one of the other above mentioned serotypes have an important advantage compared to recombinant vectors based on Ad5 with respect to clearance of the viruses by neutralising  
5 antibodies.

Also, Ad5-based vectors that have (parts of) the capsid proteins involved in immunogenic response of the host replaced by the corresponding (parts of) the capsid proteins of Ad35 or one of the other serotypes will be less, or even  
10 not, neutralised by the vast majority of human sera.

As can be seen in table I the VP/CCID50 ratio calculated from the virus particles per ml and the CCID50 obtained for each virus in the experiments was highly variable and ranged from 0.4 to 5 log. This is probably caused by different  
15 infection efficiencies of PER.C6 cells (ECACC deposit number 96022940) and by differences in replication efficiency of the viruses. Furthermore, differences in batch quantities may play a role. A high VP/CCID50 ratio means that more virus was put in the wells to obtain CPE in 5 days. As a  
20 consequence the outcome of the neutralisation study might be biased since more (inactive) virus particles could shield the antibodies. To check whether this phenomenon had taken place, the VP/CCID50 ratio was plotted against the percentage of serum samples found positive in the assay  
25 (Figure 2). The graph clearly shows that there is no negative correlation between the amount of viruses in the assay and neutralisation in serum.

## Example 2

5 Generation of Ad5 plasmid vectors for the production of  
recombinant viruses and easy manipulation of adenoviral  
genes

*pBr/Ad.Bam-rITR (ECACC deposit P97082122)*

In order to facilitate blunt end cloning of the ITR  
sequences, wild-type human adenovirus type 5 (Ad5) DNA was  
10 treated with Klenow enzyme in the presence of excess dNTPs.  
After inactivation of the Klenow enzyme and purification by  
phenol/chloroform extraction followed by ethanol  
precipitation, the DNA was digested with BamHI. This DNA  
preparation was used without further purification in a  
15 ligation reaction with pBr322 derived vector DNA prepared as  
follows: pBr322 DNA was digested with EcoRV and BamHI,  
dephosphorylated by treatment with TSAP enzyme (Life  
Technologies) and purified on LMP agarose gel (SeaPlaque  
GTG). After transformation into competent *E.coli* DH5 $\alpha$  (Life  
20 Techn.) and analysis of ampiciline resistant colonies, one  
clone was selected that showed a digestion pattern as  
expected for an insert extending from the BamHI site in Ad5  
to the right ITR.

Sequence analysis of the cloning border at the right ITR  
25 revealed that the most 3' G residue of the ITR was missing,  
the remainder of the ITR was found to be correct. Said  
missing G residue is complemented by the other ITR during  
replication.

30 *pBr/Ad.Sal-rITR (ECACC deposit P97082119)*

*pBr/Ad.Bam-rITR* was digested with BamHI and SalI. The vector  
fragment including the adenovirus insert was isolated in LMP  
agarose (SeaPlaque GTG) and ligated to a 4.8 kb SalI-BamHI  
fragment obtained from wt Ad5 DNA and purified with the  
35 Geneclean II kit (Bio 101, Inc.). One clone was chosen and  
the integrity of the Ad5 sequences was determined by

restriction enzyme analysis. Clone pBr/Ad.Sal-rITR contains adeno type 5 sequences from the SalI site at bp 16746 up to and including the rITR (missing the most 3' G residue).

5 pBr/Ad.Cla-Bam (ECACC deposit P97082117)

wt Adeno type 5 DNA was digested with ClaI and BamHI, and the 20.6 kb fragment was isolated from gel by electro-elution. pBr322 was digested with the same enzymes and purified from agarose gel by Geneclean. Both fragments were  
10 ligated and transformed into competent DH5 $\alpha$ . The resulting clone pBr/Ad.Cla-Bam was analysed by restriction enzyme digestion and shown to contain an insert with adenovirus sequences from bp 919 to 21566.

15 pBr/Ad.AflIII-Bam (ECACC deposit P97082114)

Clone pBr/Ad.Cla-Bam was linearized with EcoRI (in pBr322) and partially digested with AflIII. After heat inactivation of AflIII for 20' at 65°C the fragment ends were filled in with Klenow enzyme. The DNA was then ligated to a blunt  
20 double stranded oligo linker containing a PacI site (5'-AATTGTCTTAATTAACCGCTTAA-3'). This linker was made by annealing the following two oligonucleotides: 5'-AATTGTCTTAATTAACCGC-3' and 5'-AATTGCGGTTAATTAAGAC-3', followed by blunting with Klenow enzyme. After precipitation  
25 of the ligated DNA to change buffer, the ligations were digested with an excess PacI enzyme to remove concatameres of the oligo. The 22016 bp partial fragment containing Ad5 sequences from bp 3534 up to 21566 and the vector sequences, was isolated in LMP agarose (SeaPlaque GTG), religated and  
30 transformed into competent DH5 $\alpha$ . One clone that was found to contain the PacI site and that had retained the large adeno fragment was selected and sequenced at the 5' end to verify correct insertion of the PacI linker in the (lost) AflIII site.

35

*pBr/Ad.Bam-rITRpac#2* (ECACC deposit P97082120) and  
*pBr/Ad.Bam-rITRpac#8* (ECACC deposit P97082121)

To allow insertion of a *PacI* site near the ITR of Ad5 in  
clone *pBr/Ad.Bam-rITR* about 190 nucleotides were removed  
5 between the *ClaI* site in the *pBr322* backbone and the start  
of the ITR sequences. This was done as follows: *pBr/Ad.Bam-*  
*rITR* was digested with *ClaI* and treated with nuclease *Bal31*  
for varying lengths of time (2', 5', 10' and 15'). The  
extend of nucleotide removal was followed by separate  
10 reactions on *pBr322* DNA (also digested at the *ClaI* site),  
using identical buffers and conditions. *Bal31* enzyme was  
inactivated by incubation at 75 °C for 10', the DNA was  
precipitated and resuspended in a smaller volume TE buffer.  
To ensure blunt ends, DNAs were further treated with T4 DNA  
15 polymerase in the presence of excess dNTPs. After digestion  
of the (control) *pBr322* DNA with *Sall*, satisfactory  
degradation (~150 bp) was observed in the samples treated  
for 10' or 15'. The 10' or 15' treated *pBr/Ad.Bam-rITR*  
samples were then ligated to the above described blunted  
20 *PacI* linkers (See *pBr/Ad.AflIII-Bam*). Ligations were purified  
by precipitation, digested with excess *PacI* and separated  
from the linkers on an LMP agarose gel. After religation,  
DNAs were transformed into competent DH5 $\alpha$  and colonies  
analyzed. Ten clones were selected that showed a deletion of  
25 approximately the desired length and these were further  
analyzed by T-track sequencing (T7 sequencing kit, Pharmacia  
Biotech). Two clones were found with the *PacI* linker  
inserted just downstream of the rITR. After digestion with  
*PacI*, clone #2 has 28 bp and clone #8 has 27 bp attached to  
30 the ITR.

*pWE/Ad.AflIII-rITR* (ECACC deposit P97082116)

Cosmid vector *pWE15* (Clontech) was used to clone larger Ad5  
inserts. First, a linker containing a unique *PacI* site was  
35 inserted in the *EcoRI* sites of *pWE15* creating *pWE.pac*. To  
this end, the double stranded *PacI* oligo as described for

pBr/Ad.AflIII-BamHI was used but now with its EcoRI protruding ends. The following fragments were then isolated by electro-elution from agarose gel: pWE.pac digested with PacI, pBr/AflIII-Bam digested with PacI and BamHI and  
5 pBr/Ad.Bam-rITR#2 digested with BamHI and PacI. These fragments were ligated together and packaged using  $\lambda$  phage packaging extracts (Stratagene) according to the manufacturer's protocol. After infection into host bacteria, colonies were grown on plates and analyzed for presence of  
10 the complete insert. pWE/Ad.AflIII-rITR contains all adenovirus type 5 sequences from bp 3534 (AflIII site) up to and including the right ITR (missing the most 3' G residue).

*pBr/Ad.lITR-Sal(9.4) (ECACC deposit P97082115)*

15 Adeno 5 wt DNA was treated with Klenow enzyme in the presence of excess dNTPs and subsequently digested with SalI. Two of the resulting fragments, designated left ITR-Sal(9.4) and Sal(16.7)-right ITR, respectively, were isolated in LMP agarose (Seaplaque GTG). pBr322 DNA was  
20 digested with EcoRV and SalI and treated with phosphatase (Life Technologies). The vector fragment was isolated using the GeneClean method (BIO 101, Inc.) and ligated to the Ad5 SalI fragments. Only the ligation with the 9.4 kb fragment gave colonies with an insert. After analysis and sequencing  
25 of the cloning border a clone was chosen that contained the full ITR sequence and extended to the SalI site at bp 9462.

*pBr/Ad.lITR-Sal(16.7) (ECACC deposit P97082118)*

pBr/Ad.lITR-Sal(9.4) is digested with SalI and  
30 dephosphorylated (TSAP, Life Technologies). To extend this clone upto the third SalI site in Ad5, pBr/Ad.Cla-Bam was linearized with BamHI and partially digested with SalI. A 7.3 kb SalI fragment containing adenovirus sequences from 9462-16746 was isolated in LMP agarose gel and ligated to  
35 the SalI-digested pBr/Ad.lITR-Sal(9.4) vector fragment.

*pWE/Ad.AflIII-EcoRI*

pWE.pac was digested with ClaI and 5' protruding ends were filled using Klenow enzyme. The DNA was then digested with  
5 PacI and isolated from agarose gel. pWE/AflIII-rITR was digested with EcoRI and after treatment with Klenow enzyme digested with PacI. The large 24 kb fragment containing the adenoviral sequences was isolated from agarose gel and  
10 ligated to the ClaI-digested and blunted pWE.pac vector using the Ligation Express™ kit from Clontech. After transformation of Ultracompetent XL10-Gold cells from Stratagene, clones were identified that contained the expected insert. pWE/AflIII-EcoRI contains Ad5 sequences from bp 3534-27336.

15

*Generation of pWE/Ad.AflIII-rITRsp*

The 3' ITR in the vector pWE/Ad.AflIII-rITR does not include the terminal G-nucleotide. Furthermore, the PacI site is located almost 30 bp from the right ITR. Both these  
20 characteristics may decrease the efficiency of virus generation due to inefficient initiation of replication at the 3' ITR. Note that during virus generation the left ITR in the adapter plasmid is intact and enables replication of the virus DNA after homologous recombination.  
25 To improve the efficiency of initiation of replication at the 3' ITR, the pWE/Ad.AflIII-rITR was modified as follows: construct pBr/Ad.Bam-rITRpac#2 was first digested with PacI and then partially digested with AvrII and the 17.8 kb vector containing fragment was isolated and dephosphorylated  
30 using SAP enzyme (Boehringer Mannheim). This fragment lacks the adenosequences from nucleotide 35464 to the 3' ITR. Using DNA from pWE/Ad.AflIII-rITR as template and the primers ITR-EPH:

35

5'-CGG AAT TCT TAA TTA AGT TAA CAT CAT CAA TAA TAT ACC-3'

and

Ad101: 5'-TGA TTC ACA TCG GTC AGT GC-3'

a 630 bp PCR fragment was generated corresponding to the 3' Ad5 sequences. This PCR fragment was subsequently cloned in the vector pCR2.1 (Invitrogen) and clones containing the PCR fragment were isolated and sequenced to check correct  
5 amplification of the DNA. The PCR clone was then digested with PacI and AvrII and the 0.5 kb adeno insert was ligated to the PacI/ partial AvrII digested pBr/Ad.Bam-rITRpac#2 fragment generating pBr/Ad.Bam-rITRsp. Next this construct was used to generate a cosmid clone (as described above)  
10 that has an insert corresponding to the adenosequences 3534 to 35938. This clone was named pWE/AflIII-rITRsp.

*Generation of pWE/Ad.AflIII-rITRAE2A:*

Deletion of the E2A coding sequences from pWE/Ad.AflIII-rITR  
15 (ECACC deposit P97082116) has been accomplished as follows. The adenoviral sequences flanking the E2A coding region at the left and the right site were amplified from the plasmid pBr/Ad.Sal.rITR (ECACC deposit P97082119) in a PCR reaction with the Expand PCR system (Boehringer) according to the  
20 manufacturers protocol. The following primers were used: Right flanking sequences (corresponding Ad5 nucleotides 24033 to 25180):

ΔE2A.SnaBI: 5'-GGC GTA CGT AGC CCT GTC GAA AG-3'

ΔE2A.DBP-start: 5'-CCA ATG CAT TCG AAG TAC TTC CTT  
25 CTC CTA TAG GC-3'

The amplified DNA fragment was digested with SnaBI and NsiI (NsiI site is generated in the primer ΔE2A.DBP-start, underlined).

Left flanking sequences (corresponding Ad5 nucleotides 21557  
30 to 22442):

ΔE2A.DBP-stop: 5'-CCA ATG CAT ACG GCG CAG ACG G-3'

ΔE2A.BamHI: 5'-GAG GTG GAT CCC ATG GAC GAG-3'

The amplified DNA was digested with BamHI and NsiI (NsiI site is generated in the primer ΔE2A.DBP-stop, underlined).

35 Subsequently, the digested DNA fragments were ligated into

SnaBI/BamHI digested pBr/Ad.Sal-rITR. Sequencing confirmed the exact replacement of the DBP coding region with a unique NsiI site in plasmid pBr/Ad.Sal-rITRAE2A. The unique NsiI site can be used to introduce an expression cassette for a gene to be transduced by the recombinant vector.

The deletion of the E2A coding sequences was performed such that the splice acceptor sites of the 100K encoding L4-gene at position 24048 in the top strand was left intact. In addition, the poly adenylation signals of the original E2A-RNA and L3-RNAs at the left hand site of the E2A coding sequences were left intact. This ensures proper expression of the L3-genes and the gene encoding the 100K L4-protein during the adenovirus life cycle.

Next, the plasmid pWE/Ad.AflIII-rITRAE2A was generated. The plasmid pBr/Ad.Sal-rITRAE2A was digested with BamHI and SpeI. The 3.9-Kb fragment in which the E2A coding region was replaced by the unique NsiI site was isolated. The pWE/Ad.AflIII-rITR was digested with BamHI and SpeI. The 35 Kb DNA fragment, from which the BamHI/SpeI fragment containing the E2A coding sequence was removed, was isolated. The fragments were ligated and packaged using  $\lambda$  phage-packaging extracts according to the manufacturer protocol (Stratagene), yielding the plasmid pWE/Ad.AflIII-rITRAE2A.

This cosmid clone can be used to generate adenoviral vectors that are deleted for E2A by cotransfection of PacI digested DNA together with digested adapter plasmids onto packaging cells that express functional E2A gene product.

### 30 Construction of adapter plasmids

The absence of sequence overlap between the recombinant adenovirus and E1 sequences in the packaging cell line is essential for safe, RCA-free generation and propagation of new recombinant viruses. The adapter plasmid pMLPI.TK (described in PCT/NL96/00244) is an example of an adapter plasmid designed for use according to the invention in



combination with the improved packaging cell lines of the invention. This plasmid was used as the starting material to make a new vector in which nucleic acid molecules comprising specific promoter and gene sequences can be easily

5 exchanged.

First, a PCR fragment was generated from pZipΔMo+PyF101(N<sup>+</sup>) template DNA (described in PCT/NL96/00195) with the following primers: LTR-1: 5'-CTG TAC GTA CCA GTG CAC TGG CCT AGG CAT GGA AAA ATA CAT AAC TG-3' and LTR-2: 5'-GCG GAT CCT  
10 TCG AAC CAT GGT AAG CTT GGT ACC GCT AGC GTT AAC CGG GCG ACT CAG TCA ATC G-3'. Pwo DNA polymerase (Boehringer Mannheim) was used according to manufacturers protocol with the following temperature cycles: once 5' at 95°C; 3' at 55°C; and 1' at 72°C, and 30 cycles of 1' at 95°C, 1' at 60°C, 1'  
15 at 72°C, followed by once 10' at 72°C. The PCR product was then digested with BamHI and ligated into pMLP10 (Levrero et al., 1991) vector digested with PvuII and BamHI, thereby generating vector pLTR10. This vector contains adenoviral sequences from bp 1 up to bp 454 followed by a promoter  
20 consisting of a part of the Mo-MuLV LTR having its wild-type enhancer sequences replaced by the enhancer from a mutant polyoma virus (PyF101). The promoter fragment was designated L420. Next, the coding region of the murine HSA gene was inserted. pLTR10 was digested with BstBI followed by Klenow  
25 treatment and digestion with NcoI. The HSA gene was obtained by PCR amplification on pUC18-HSA (Kay et al., 1990) using the following primers: HSA1, 5'-GCG CCA CCA TGG GCA GAG CGA TGG TGG C-3' and HSA2, 5'-GTT AGA TCT AAG CTT GTC GAC ATC GAT CTA CTA ACA GTA GAG ATG TAG AA-3'. The 269 bp amplified  
30 fragment was subcloned in a shuttle vector using the NcoI and BglII sites. Sequencing confirmed incorporation of the correct coding sequence of the HSA gene, but with an extra TAG insertion directly following the TAG stop codon. The coding region of the HSA gene, including the TAG duplication  
35 was then excised as a NcoI(sticky)-SalI(blunt) fragment and

cloned into the 3.5 kb NcoI(sticky)/BstBI(blunt) fragment from pLTR10, resulting in pLTR-HSA10.

Finally, pLTR-HSA10 was digested with EcoRI and BamHI after which the fragment containing the left ITR, packaging  
5 signal, L420 promoter and HSA gene was inserted into vector pMLPI.TK digested with the same enzymes and thereby replacing the promoter and gene sequences. This resulted in the new adapter plasmid pAd/L420-HSA that contains  
10 convenient recognition sites for various restriction enzymes around the promoter and gene sequences. SnaBI and AvrII can be combined with HpaI, NheI, KpnI, HindIII to exchange promoter sequences, while the latter sites can be combined with the ClaI or BamHI sites 3' from HSA coding region to replace genes in this construct.

15 Another adapter plasmid that was designed to allow easy exchange of nucleic acid molecules was made by replacing the promoter, gene and poly A sequences in pAd/L420-HSA with the CMV promoter, a multiple cloning site, an intron and a poly-A signal. For this purpose, pAd/L420-HSA was digested with  
20 AvrII and BglII followed by treatment with Klenow to obtain blunt ends. The 5.1 kb fragment with pBr322 vector and adenoviral sequences was isolated and ligated to a blunt 1570 bp fragment from pcDNA1/amp (Invitrogen) obtained by digestion with HhaI and AvrII followed by treatment with T4  
25 DNA polymerase. This adapter plasmid was named pAd5/CLIP. To enable removal of vector sequences from the left ITR in pAd5/Clip, this plasmid was partially digested with EcoRI and the linear fragment was isolated. An oligo of the sequence 5' TTAAGTCGAC-3' was annealed to itself resulting  
30 in a linker with a SalI site and EcoRI overhang. The linker was ligated to the partially digested pAd5/Clip vector and clones were selected that had the linker inserted in the EcoRI site 23 bp upstream of the left adenovirus ITR in pAd5/Clip resulting in pAd5/Clipsal. Likewise, the EcoRI  
35 site in pAd5/Clip has been changed to a PacI site by insertion of a linker of the sequence 5'-

AATTGTCTTAATTAACCGCAATT-3'. The pAd5/Clip vector was partially digested with EcoRI, dephosphorylated and ligated to the PacI linker with EcoRI overhang. The ligation mixture was digested with PacI to remove concatamers, isolated from agarose gel and religated. The resulting vector was named pAd5/Clippac. These changes enable more flexibility to liberate the left ITR from the plasmid vector sequences. The vector pAd5/L420-HSA was also modified to create a Sall or PacI site upstream of the left ITR. Hereto pAd5/L420-HSA was digested with EcoRI and ligated to the above described PacI linker. The ligation mixture was digested with PacI and religated after isolation of the linear DNA from agarose gel to remove concatamerised linkers. This resulted in adapter plasmid pAd5/L420-HSApac. This construct was used to generate pAd5/L420-HSAsal as follows: pAd5/L420-HSApac was digested with ScaI and BsrGI and the vector fragment was ligated to the 0.3 kb fragment isolated after digestion of pAd5/Clipsal with the same enzymes.

## 20 *Generation of adapter plasmids pAdMire and pAdApt*

To create an adapter plasmid that only contains a polylinker sequence and no promoter or polyA sequences, pAd5/L420-HSApac was digested with AvrII and BglII. The vector fragment was ligated to a linker oligonucleotide digested with the same restriction enzymes. The linker was made by annealing oligos of the following sequence:

PLL-1: 5'- GCC ATC CCT AGG AAG CTT GGT ACC GGT GAA TTC GCT AGC GTT AAC GGA TCC TCT AGA CGA GAT CTG G-3' and  
PLL-2: 5'- CCA GAT CTC GTC TAG AGG ATC CGT TAA CGC TAG CGA ATT CAC CGG TAC CAA GCT TCC TAG GGA TGG C-3'.

The annealed linkers were digested with AvrII and BglII and separated from small ends by column purification (Qiaquick nucleotide removal kit) according to manufacturerers recommendations. The linker was then ligated to the AvrII/BglII digested pAd5/L420-HSApac fragment. A clone,

named AdMire, was selected that had the linker incorporated and was sequenced to check the integrity of the insert. Adapter plasmid AdMire enables easy insertion of complete expression cassettes.

- 5 An adapter plasmid containing the human CMV promoter that mediates high expression levels in human cells was constructed as follows: pAd5/L420-HSAPac was digested with AvrII and 5' protruding ends were filled in using Klenow enzyme. A second digestion with HindIII resulted in removal  
10 of the L420 promoter sequences. The vector fragment was isolated and ligated to a PCR fragment containing the CMV promoter sequence. This PCR fragment was obtained after amplification of CMV sequences from pCMVLacI (Stratagene) with the following primers:
- 15 CMVplus: 5'-GATCGGTACCACTGCAGTGGTCAATATTGGCCATTAGCC-3' and  
CMVminA: 5'-GATCAAGCTTCCAATGCACCGTTCCCGGC-3'.
- The PCR fragment was first digested with PstI (underlined in CMVplus) after which the 3'-protruding ends were removed by treatment with T4 DNA polymerase. Then the DNA was digested  
20 with HindIII (underlined in CMVminA) and ligated into the above described pAd5/L420-HSAPac vector fragment digested with AvrII and HindIII. The resulting plasmid was named pAd5/CMV-HSAPac. This plasmid was then digested with HindIII and BamHI and the vector fragment was isolated and ligated  
25 to the polylinker sequence obtained after digestion of AdMire with HindIII and BglII. The resulting plasmid was named pAdApt. Adapter plasmid pAdApt contains nucleotides -735 to +95 of the human CMV promoter (Boshart et al., 1985). A second version of this adapter plasmid containing a SalI  
30 site in place of the PacI site upstream of the left ITR was made by inserting the 0.7 kb ScaI-BsrGI fragment from pAd5/Clipsal into pAdApt digested with ScaI and partially digested with BsrGI. This clone was named pAdApt.sal.

*Generation of recombinant adenoviruses based on Ad5*

RCA free recombinant adenoviruses can be generated very efficiently using the above described adapter plasmids and the pWe/Ad.AflIII-rITR or pWE/Ad.AflIII-rITrsp constructs.

- 5 Generally, the adapter plasmid containing the desired transgene in the desired expression cassette is digested with suitable enzymes to liberate the insert from vector sequences at the 3' and/or at the 5' end. The adenoviral complementation plasmids pWE/Ad.AflIII-rITR or pWE/Ad.AflIII-rITrsp are digested with PacI to liberate the adeno  
10 sequences from the vector plasmids. As a non-limiting example the generation of AdApt-LacZ is described. Adapter plasmid pAdApt-LacZ was generated as follows. The E.coli LacZ gene was amplified from the plasmid pMLP.nlsLacZ (EP  
15 95-202 213) by PCR with the primers 5'-  
GGGGTGGCCAGGTACCTCTAGGCTTTTGCAA-3' and 5'-  
GGGGGGATCCATAAACAAGTTCAGAAATCC-3'. The PCR reaction was performed with Ex Taq (Takara) according to the suppliers protocol at the following amplification program: 5 minutes  
20 94°C, 1 cycle; 45 seconds 94°C and 30 seconds 60°C and 2 minutes 72°C, 5 cycles; 45 seconds 94°C and 30 seconds 65°C and 2 minutes 72°C, 25 cycles; 10 minutes 72; 45 seconds 94°C and 30 seconds 60°C and 2 minutes 72°C, 5 cycles, 1 cycle. The PCR product was subsequently digested with KpnI  
25 and BamHI and the digested DNA fragment was ligated into KpnI/BamHI digested pcDNA3 (Invitrogen), giving rise to pcDNA3.nlsLacZ. Construct pcDNA3.nlsLacZ was then digested with KpnI and BamHI and the 3 kb LacZ fragment was isolated from gel using the geneclean spin kit (Bio 101, Inc.).  
30 pAdApt was also digested with KpnI and BamHI and the linear vector fragment was isolated from gel as above. Both isolated fragments were ligated and one clone containing the LacZ insert was selected. Construct pAdApt-LacZ was digested with SalI, purified by the geneclean spin kit and  
35 subsequently digested with PacI. pWE/Ad.AflIII-rITrsp was digested with PacI. Both digestion mixtures were treated for

30' by 65 °C to inactivate the enzymes. Samples were put on gel to estimate the concentration.  $2.5 \times 10^6$  PER.C6 cells (ECACC deposit number 96022940) were seeded in T25 flasks in DMEM with 10% FCS and 10mM MgCl. The next day four microgram of each plasmid was transfected into PER.C6 cells (ECACC deposit number 96022940) using lipofectamine transfection reagent (Life Technologies Inc.) according to instructions of the manufacturer. The next day the medium was replaced by fresh culture medium and cells were further cultured at 37° C, 10% CO<sub>2</sub>. Again 24 hrs. later cells were trypsinised, seeded into T80 flasks and cultured at 37°C, 10% CO<sub>2</sub>. Full CPE was obtained 6 days after seeding in the T80 flask. Cells were harvested in the medium and subjected to one freeze/thaw cycle. The crude lysate obtained this way was used to plaque purify the mixture of viruses. Ten plaques were picked, expanded in a 24 well plate and tested for LacZ expression following infection of A549 cells. Viruses from all ten plaques expressed LacZ.

### 20 Example 3

#### Generation of chimeric recombinant adenoviruses

##### *Generation of hexon chimeric Ad5-based adenoviruses*

25 Neutralising antibodies in human serum are mainly directed to the hexon protein and to a lesser extent to the penton protein. Hexon proteins from different serotypes show highly variable regions present in loops that are predicted to be exposed at the outside of the virus (Athappilly et al., 1994; J. Mol. Biol. 242, 430-455). Most type specific epitopes have been mapped to these highly variable regions (Toogood et al., 1989; J. Gen Virol. 70, 3203-3214). Thus replacement of (part of ) the hexon sequences with corresponding sequences from a different serotype is an effective strategy to circumvent (pre-existing) neutralising

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antibodies to Ad5. Hexon coding sequences of adenovirus serotype 5 are located between nucleotides 18841 and 21697. To facilitate easy exchange of hexon coding sequences from alternative adenovirus serotypes into the adenovirus serotype 5 backbone, first a shuttle vector was generated. This subclone, coded pBr/Ad.Eco-PmeI, was generated by first digesting plasmid pBr322 with EcoRI and EcoRV and inserting the 14 kb PmeI-EcoRI fragment from pWE/Ad.AflIII-Eco. In this shuttle vector a deletion was made of a 1430 bp SanDI fragment by digestion with SanDI and religation to give pBr/Ad.Eco-PmeI  $\Delta$ SanDI. The removed fragment contains unique SpeI and MunI sites. From pBr/Ad.Eco-PmeI  $\Delta$ SanDI the adenovirus serotype 5 DNA encoding hexon was deleted. Hereto, the hexon flanking sequences were PCR amplified and linked together thereby generating unique restriction sites replacing the hexon coding region. For these PCR reactions four different oligonucleotides were required:  $\Delta$ hex1- $\Delta$ hex4.

$\Delta$ hex1: 5'- CCT GGT GCT GCC AAC AGC-3'

$\Delta$ hex2: 5'- CCG GAT CCA CTA GTG GAA AGC GGG CGC GCG-3'

$\Delta$ hex3: 5'- CCG GAT CCA ATT GAG AAG CAA GCA ACA TCA ACA AC-3'

$\Delta$ hex4: 5'- GAG AAG GGC ATG GAG GCT G-3'

The amplified DNA product of  $\pm$  1100 bp obtained with oligonucleotides  $\Delta$ hex1 and  $\Delta$ hex2 was digested with BamHI and FseI. The amplified DNA product of  $\pm$  1600 bp obtained with oligonucleotides  $\Delta$ hex3 and  $\Delta$ hex4 was digested with BamHI and SbfI. These digested PCR fragments were subsequently purified from agarose gel and in a tri-part ligation reaction using T4 ligase enzyme linked to pBr/Ad.Eco-PmeI  $\Delta$ SanDI digested with FseI and SbfI. The resulting construct was coded pBr/Ad.Eco-PmeI  $\Delta$ Hexon. This construct was sequenced in part to confirm the correct nucleotide sequence and the presence of unique restriction sites MunI and SpeI. pBr/Ad.Eco-PmeI  $\Delta$ Hexon serves as a shuttle vector to introduce heterologous hexon sequences amplified from virus DNA from different serotypes using primers that introduce the unique

restriction sites *MunI* and *SpeI* at the 5' and 3' ends of the hexon sequences respectively. To generate Ad5-based vectors that contain hexon sequences from the serotypes to which healthy individuals have no, or very low, titers of NAB the hexon sequences of Ad35, Ad34, Ad26 and Ad48 were amplified using the following primers:

Hex-up2: 5'-GACTAGTCAAGATGGCYACCCCHTCGATGATG-3' and

Hex-do2: 5'-GCTGGCCAATTGTTATGTKGKGCGTTRCCGGC-3'.

These primers were designed using the sequences of published hexon coding regions (for example hexon sequences of Ad2, Ad3, Ad4, Ad5, Ad7, Ad16, Ad40 and Ad41 can be obtained at Genbank). Degenerated nucleotides were incorporated at positions that show variation between serotypes.

PCR products were digested with *SpeI* and *MunI* and cloned into the pBr/Ad.Eco-PmeΔHexon construct digested with the same enzymes.

The hexon modified sequences were subsequently introduced in the construct pWE/Ad.AflIII-rITR by exchange of the *AscI* fragment generating pWE/Ad.AflIII-rITRHexXX where XX stands for the serotype used to amplify hexon sequences.

The pWE/Ad.AflIII-rITRHexXX constructs are then used to make viruses in the same manner as described above for Ad5 recombinant viruses.

## 25 *Generation of penton chimeric Ad5-based recombinant viruses*

The adenovirus type 5 penton gene is located between sequences 14156 and 15869. Penton base is the adenovirus capsid protein that mediates internalisation of the virus into the target cell. At least some serotypes (type C and B) have been shown to achieve this by interaction of an RGD sequence in penton with integrins on the cell surface. However, type F adenoviruses do not have an RGD sequence and for most viruses of the A and D group the penton sequence is not known. Therefore, penton may be involved in target cell specificity. Furthermore, as a capsid protein, the penton



protein is involved in the immunogenicity of the adenovirus (Gahery-Segard et al., 1998). Therefore, replacement of Ad5 penton sequences with penton sequences from serotypes to which no or low titers of NAB exist in addition to

- 5 replacement of the hexon sequences will prevent clearance of the adenoviral vector more efficiently than replacement of hexon alone. Replacement of penton sequences may also affect infection specificity.

To be able to introduce heterologous penton sequences in Ad5  
10 we made use of the plasmid-based system described above.

First a shuttle vector for penton sequences was made by insertion of the 7.2 kb NheI-EcoRV fragment from construct pWE/Ad.AflIII-EcoRI into pBr322 digested with the same enzymes. The resulting vector was named pBr/XN. From this  
15 plasmid Ad5 penton sequences were deleted and replaced by unique restriction sites that are then used to introduce new penton sequences from other serotypes. Hereto, the left flanking sequences of penton in pBr/XN were PCR amplified using the following primers:

- 20 DP5-F: 5'- CTG TTG CTG CTG CTA ATA GC-3' and  
DP5-R: 5'- CGC GGA TCC TGT ACA ACT AAG GGG AAT ACA AG-3'  
DP5-R has an BamHI site (underlined) for ligation to the right flanking sequence and also introduces a unique BsrGI site (bold face) at the 5'-end of the former Ad5 penton  
25 region.

The right flanking sequence was amplified using:

- DP3-F: 5'-CGC GGA TCC CTT AAG GCA AGC ATG TCC ATC CTT-3' and  
DP3-3R: 5'- AAA ACA CGT TTT ACG CGT CGA CCT TTC-3'  
DP3-F has an BamHI site (underlined) for ligation to the  
30 left flanking sequence and also introduces a unique AflIII site (bold face) at the 3'-end of the former Ad5 penton region.

- The two resulting PCR fragments were digested with BamHI and ligated together. Then this ligation mixture was digested  
35 with AvrII and BglII. pBr/XN was also digested with AvrII

and BglII and the vector fragment was ligated to the digested ligated PCR fragments. The resulting clone was named pBr/Ad.Δpenton. Penton coding sequences from Ad35, Ad34, Ad26 and Ad48 were PCR amplified such that the 5' and 3' ends contained the BsrGI and AflII sites respectively. Hereto, the following primers were used:

For Ad34 and Ad35:

P3-for: 5'-GCT CGA TGT ACA ATG AGG AGA CGA GCC GTG CTA-3'

P3-rev: 5'-GCT CGA CTT AAG TTA GAA AGT GCG GCT TGA AAG-3'

For Ad26 and Ad48:

P17F: 5'-GCT CGA TGT ACA ATG AGG CGT GCG GTG GTG TCT TC-3'

P17R: 5'-GCT CGA CTT AAG TTA GAA GGT GCG ACT GGA AAG C-3'

Amplified pcr products were digested with BfrI and BsrGI and cloned into pBr/Ad.Δpenton digested with the same enzymes. Introduction of these heterologous penton sequences in pBr/Ad.Δpenton generated constructs named pBr/Ad.pentonXX where XX represents the number of the serotype corresponding to the serotype used to amplify the inserted penton sequences. Subsequently the new penton sequences were introduced in the a pWE/Ad.AflIII-rITR vector having a modified hexon. For example penton sequences from Ad35 were introduced in the construct pWE/Ad.AflIII-rITRHex35 by exchange of the common FseI fragment. Other combinations of penton and hexon sequences were also made. Viruses with modified hexon and penton sequences were made as described above using cotransfection with an adapter plasmid on PER.C6 cells (ECACC deposit number 96022940). In addition, penton sequences were introduced in the pWE/Ad.AflIII-rITR construct. The latter constructs contain only a modified penton and viruses generated from these constructs will be used to study the contribution of penton sequences to the neutralisation of adenoviruses and also for analysis of possible changes in infection efficiency and specificity.

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*Generation of fiber chimeric Ad5-based viruses*

Adenovirus infection is mediated by two capsid proteins fiber and penton. Binding of the virus to the cells is achieved by interaction of the protruding fiber protein with a receptor on the cell surface. Internalisation then takes place after interaction of the penton protein with integrins on the cell surface. At least some adenovirus from subgroup C and B have been shown to use a different receptor for cell binding and therefor have different infection efficiencies on different cell types. Thus it is possible to change the infection spectrum of adenoviruses by changing the fiber in the capsid. The fiber coding sequence of adenovirus serotype 5 is located between nucleotides 31042 and 32787. To remove the adenovirus serotype 5 DNA encoding fiber we started with construct pBr/Ad.Bam-rITR. First a NdeI site was removed from this construct. For this purpose, pBr322 plasmid DNA was digested with NdeI after which protruding ends were filled using Klenow enzym. This pBr322 plasmid was then religated, digested with NdeI and transformed into *E.coli* DH5 $\alpha$ . The obtained pBr/ $\Delta$ NdeI plasmid was digested with ScaI and SalI and the resulting 3198 bp vector fragment was ligated to the 15349 bp ScaI-SalI fragment derived from pBr/Ad.BamrITR, resulting in plasmid pBr/Ad.Bam-rITR $\Delta$ NdeI which hence contained a unique NdeI site. Next a PCR was performed with oligonucleotides

NY-up:  
5'- CGA CAT ATG TAG ATG CAT TAG TTT GTG TTA TGT TTC AAC GTG-3'

and

NY-down:  
5'-GGA GAC CAC TGC CAT GTT-3'

During amplification, both a NdeI (bold face) and a NsiI restriction site (underlined) were introduced to facilitate cloning of the amplified fiber DNAs. Amplification consisted of 25 cycles of each 45 sec. at 94°C, 1 min. at 60°C, and 45 sec. at 72°C. The PCR reaction contained 25 pmol of oligonucleotides NY-up or NY-down, 2mM dNTP, PCR buffer with

1.5 mM MgCl<sub>2</sub>, and 1 unit of Elongase heat stable polymerase (Gibco, The Netherlands). One-tenth of the PCR product was run on an agarose gel which demonstrated that the expected DNA fragment of  $\pm$  2200 bp was amplified. This PCR fragment was subsequently purified using Geneclean kit system (Bio101 Inc.). Then, both the construct pBr/Ad.Bam-rITRANdeI as well as the PCR product were digested with restriction enzymes NdeI and SbfI. The PCR fragment was subsequently cloned using T4 ligase enzyme into the NdeI and SbfI digested pBr/Ad.Bam-rITRANdeI, generating pBr/Ad.BamRA $\Delta$ Fib. This plasmid allows insertion of any PCR amplified fiber sequence through the unique NdeI and NsiI sites that are inserted in place of the removed fiber sequence. Viruses can be generated by a double homologous recombination in packaging cells described in patent No. PCT/NL96/00244 using an adapter plasmid, construct pBr/Ad.AflIII-EcoRI digested with PacI and EcoRI and a pBr/Ad.BamRA $\Delta$ Fib construct in which heterologous fiber sequences have been inserted. To increase the efficiency of virus generation, the construct pBr/Ad.BamRA $\Delta$ Fib was modified to generate a PacI site flanking the right ITR. Hereto, pBr/Ad.BamRA $\Delta$ Fib was digested with AvrII and the 5 kb adenofragment was isolated and introduced into the vector pBr/Ad.Bam-rITR.pac#8 described above replacing the corresponding AvrII fragment. The resulting construct was named pBr/Ad.BamRA $\Delta$ Fib.pac. Once a heterologous fiber sequence is introduced in pBr/Ad.BamRA $\Delta$ Fib.pac, the fiber modified right hand adenovirus clone is introduced into a large cosmid clone as described above for pWE/Ad.AflIII-rITR. Such a large cosmid clone allows generation of adenovirus by only one homologous recombination. Ad5-based viruses with modified fibers have been made and described (nos. 98204482.8 and 99200624.7). In addition, hexon and penton sequences from serotypes from this invention are combined with the desired fiber sequences to generate viruses which infect the target cell of choice

very efficiently. For example smooth muscle cells, endothelial cells or synoviocytes all from human origin are very well infected with Ad5 based viruses with a fiber from subgroup B viruses especially adenovirus type 16.

- 5 The above described examples in which specific sequences can be deleted from the Ad5 backbone in the plasmids and replaced by corresponding sequences from other serotypes clearly show the flexibility of the system. It is evident that by the methods described above any combination of
- 10 capsid gene from different serotypes can be made. Thus, chimeric recombinant Ad5-based adenoviruses are designed with desired hexon and penton sequences making the virus less sensitive for neutralisation and with desired fiber sequences allowing efficient infection in specific target
- 15 tissues.

#### Example 4

##### 20 Construction of a plasmid-based system to generate Ad35 recombinant viruses

Partial restriction maps of Ad35 have been published previously (Valderrama-Leon et al., 1985; Kang et al., 1989; Li et al. 1991). An example of a functional plasmid-based

25 system to generate recombinant adenoviruses based on Ad35 consists of the following elements:

1. An adapter plasmid comprising a left ITR and packaging sequences derived from Ad35 and at least one restriction site for insertion of an heterologous expression cassette and lacking E1 sequences. Furthermore, the adapter
- 30 plasmid contains Ad35 sequences 3' from the E1B coding region including the pIX promoter and coding sequences sufficient to mediate homologous recombination of the adapter plasmid with a second nucleotide.
- 35 2. A second nucleotide comprising sequences homologous to the adapter plasmid and Ad35 sequences necessary for the

replication and packaging of the recombinant virus, that is early, intermediate and late genes that are not present in the packaging cell.

3. A packaging cell providing at least functional E1  
5 proteins capable of complementing the E1 function of Ad35.

Ad35 DNA was isolated from a purified virus batch as follows. To 100  $\mu$ l of virus stock (Ad35:  $3.26 \times 10^{12}$  VP/ml)  
10 10 $\mu$ l 10x DNase buffer (130 mM Tris-HCl pH7.5; 1,2 M CaCl<sub>2</sub>; 50mM MgCl<sub>2</sub>) was added. After addition of 10  $\mu$ l 10mgr/ml DNase I (Roche Diagnostics) the mixture was incubated for 1 hr. at 37°C. Following addition of 2.5 $\mu$ l 0.5M EDTA, 3.2 $\mu$ l 20% SDS and 1.5 $\mu$ l ProteinaseK (Roche Diagnostics; 20mgr/ml)  
15 samples were incubated at 50°C for 1 hr. Next, the viral DNA was isolated using the Geneclean spin kit (Bio101 Inc.) according to the manufacturers instructions. DNA was eluted from the spin column with 25  $\mu$ l sterile MilliQ water. In the following sizes of DNA fragments and fragment  
20 numbering will be used according to Kang et al. (1989). Ad35 DNA was digested with EcoRI and the three fragments (approximately 22.3 (A), 7.3 (B) and 6 kb (C)) were isolated from gel using the Geneclean kit (Bio101, Inc.). pBr322 was digested with EcoRI or with EcoRI and EcoRV and digested  
25 fragments were isolated from gel and dephosphorylated with Tsap enzyme (Gibco BRL). Next, the 6 kb Ad35 C fragment was ligated to the pBr322xEcoRI fragment and the ITR-containing Ad35 fragment (EcoRI-B) was ligated to the pBr322xEcoRI/EcoRV fragment. Ligations were incubated at  
30 16°C overnight and transformed into DH5 $\alpha$  competent bacteria (Life Techn.). Minipreps of obtained colonies were analysed for correct insertion of the Ad35 fragments by restriction analysis. Both the 6 kb and the 7.3 kb Ad35 fragment were found to be correctly inserted in pBr322. The 6kb fragment  
35 was isolated in both orientations pBr/Ad35-Eco6.0<sup>+</sup> and pBr/Ad35-Eco6.0<sup>-</sup> whereby the + stands for 5' to 3'

orientation relative to pBr322. The clone with the 7.3 kb Ad35 B insert, named pBr/Ad35-Eco7.3 was partially sequenced to check correct ligation of the 3' ITR. It was found that the ITR had the sequence 5'- CATCATCAAT...-3' in the lower strand. Then pBr/Ad35-Eco7.3 was extended to the 5' end by insertion of the 6kb Ad35 fragment. Hereto, pBr/Ad35-Eco7.3 was digested with EcoRI and dephosphorylated. The fragment was isolated from gel and ligated to the 6kb Ad35 EcoRI fragment. After transformation clones were tested for correct orientation of the insert and one clone was selected, named pBr/Ad35-Eco13.3.

This clone is then extended with the ~5.4 kb SalI D fragment obtained after digestion of wt Ad35 with SalI. Hereto, the SalI site in the pBr322 backbone is removed by partial digestion of pBr/Ad35-Eco13.3 with SalI, filling in of the sticky ends by Klenow treatment and religation. One clone is selected that contains a single SalI site in the adenoviral insert. This clone, named pBr $\Delta$ sal/Ad35-Eco13.3 is then linearised with AatII which is present in the pBr322 backbone and ligated to a SalI linker with AatII complementary ends. The DNA is then digested with excess SalI and the linear fragment is isolated and ligated to the 5.4 kb SalI-D fragment from Ad35. One clone is selected that contains the SalI fragment inserted in the correct orientation in pBr/Ad35-Eco13.3. The resulting clone, pBr/Ad35.Sal2-rITR contains the 3' ~17 kb of Ad35 including the right ITR. To enable liberation of the right ITR from the vector sequences at the time of virus generation, a NotI site flanking the right ITR is introduced by PCR.

The Ad35 EcoRI-A fragment of 22.3 kb was also cloned in pBr322xEcoRI/EcoRV. One clone, named pBr/Ad35-EcoA3', was selected that apparently had a deletion of approximately 7kb of the 5' end. It did contain the SalI site at 9.4 kb in Ad35 wt DNA and approximately 1.5 kb of sequences upstream.

Using this SalI site and the unique NdeI site in the pBr322 backbone this clone is extended to the 5' end by insertion

of an approximately 5 kb Ad35 fragment 5' from the first SalI in Ad35 in such a way that a NotI restriction site is created at the 5' end of the Ad35 by insertion of a linker. This clone, named pBr/Ad35.pIX-EcoA does not contain the left end sequences (ITR, packaging sequences and E1) and at the 3' end it has approximately 3.5 kb overlap with clone pBr/Ad35.Sal2-rITR.

To create an adapter plasmid, Ad35 was digested with SalI and the left end B fragment of ~9.4 kb was isolated. pBr322 was digested with EcoRV and SalI, isolated from gel and dephosphorylated with Tsap enzyme. Both fragments are ligated and clones with correct insertion and correct sequence of the left ITR are selected. To enable liberation of the left ITR from the vector sequences at the time of virus generation, a NotI site flanking the left ITR is introduced by PCR. From this clone the E1 sequences are deleted and replaced by a polylinker sequence using PCR. The polylinker sequence is used to introduce an expression cassette for a gene of choice.

Recombinant Ad35 clones are generated by transfection of PER.C6 cells with the adapter plasmid, pBr/Ad35.pIX-EcoA and pBr/Ad35.Sal2-rITR as shown in figure 3. Homologous recombination gives rise to recombinant viruses.

Example 5

25     ,

The prevalence of neutralizing activity (NA) to Ad35 is low in human sera from different geographic locations .

In example 1 we have described the analysis of neutralizing activity (NA) in human sera from one location in Belgium. Strikingly, of a panel of 44 adenovirus serotypes tested, one serotype, Ad35, was not neutralized in any of the 100 sera assayed. In addition, a few serotypes, Ad26, Ad34 and Ad48 were found to be neutralized in 8%, or less, of the sera tested. This analysis was further extended to other serotypes of adenovirus not previously tested and,



using a selection of serotypes from the first screen, was also extended to sera from different geographic locations.

Hereto, adenoviruses were propagated, purified and tested for neutralization in the CPE-inhibition assay as described in example 1. Using the sera from the same batch as in example 1, adenovirus serotypes 7B, 11, 14, 18 and 44/1876 were tested for neutralization. These viruses were found to be neutralized in respectively 59, 13, 30, 98 and 54 % of the sera. Thus, of this series Ad11 is neutralized with a relatively low frequency.

Since it is known that the frequency of isolation of adenovirus serotypes from human tissue as well as the prevalence of NA to adenovirus serotypes may differ on different geographic locations, we further tested a selection of the adenovirus serotypes against sera from different places. Human sera were obtained from two additional places in Europe (Bristol, UK and Leiden, The Netherlands) and from two places in the United States (Stanford, CA and Great Neck, NY). Adenoviruses that were found to be neutralized in 20% or less of the sera in the first screen, as well as Ad2, Ad5, Ad27, Ad30, Ad38, Ad43, were tested for neutralization in sera from the UK. The results of these experiments are presented in Figure 4. Adenovirus serotypes 2 and 5 were again neutralized in a high percentage of human sera. Furthermore, some of the serotypes that were neutralized in a low percentage of sera in the first screen are neutralized in a higher percentage of sera from the UK, e.g. Ad26 (7% vs. 30%), Ad28 (13% vs. 50%), Ad34 (5% vs. 27%) and Ad48 (8% vs. 32%). Neutralizing activity against Ad11 and Ad49 that were found in a relatively low percentage of sera in the first screen, are found in an even lower percentage of sera in this second screen (13% vs. 5% and 20% vs. 11% respectively). Serotype Ad35 that was not neutralized in any of the sera in the first screen, was now found to be neutralized in a low percentage (8%) of sera from the UK. The prevalence of NA in

human sera from the UK is the lowest to serotypes Ad11 and Ad35.

For further analysis, sera were obtained from two locations in the US (Stanford, CA and Great Neck, NY) and from the Netherlands (Leiden). Figure 5 presents an overview of data obtained with these sera and the previous data. Not all viruses were tested in all sera, except for Ad5, Ad11 and Ad35. The overall conclusion from this comprehensive screen of human sera is that the prevalence of neutralizing activity to Ad35 is the lowest of all serotypes throughout the western countries: on average 7% of the human sera contain neutralizing activity (5 different locations). Another B-group adenovirus, Ad11 is also neutralized in a low percentage of human sera (average 11% in sera from 5 different locations). Adenovirus type 5 is neutralized in 56% of the human sera obtained from 5 different locations. Although not tested in all sera, D-group serotype 49 is also neutralized with relatively low frequency in samples from Europe and from one location of the US (average 14%).

In the above described neutralization experiments a serum is judged non-neutralizing when in the well with the highest serum concentration the maximum protection of CPE is 40% compared to the controls without serum. The protection is calculated as follows:

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$$\% \text{ protection} = \frac{\text{OD corresponding well} - \text{OD virus control}}{\text{OD non-infected control} - \text{OD virus control}} \times 100 \%$$

As described in example 1, the serum is plated in five different dilutions ranging from 4x to 64x diluted. Therefore, it is possible to distinguish between low titers (i.e. neutralization only in the highest serum concentrations) and high titers of NA (i.e. also neutralization in wells with the lowest serum concentration). Of the human sera used in our screen that

were found to contain neutralizing activity to Ad5, 70% turned out to have high titers whereas of the sera that contained NA to Ad35, only 15% had high titers. Of the sera that were positive for NA to Ad11 only 8% had high titers. For Ad49 this was 5%. Therefore, not only is the frequency of NA to Ad35, Ad11 and Ad49 much lower as compared to Ad5, but of the sera that do contain NA to these viruses, the vast majority has low titers. Adenoviral vectors based on Ad11, Ad35 or Ad49 have therefore a clear advantage over Ad5 based vectors when used as gene therapy vehicles or vaccination vectors *in vivo* or in any application where infection efficiency is hampered by neutralizing activity.

In the following examples the construction of a vector system for the generation of safe, RCA-free Ad35-based vectors is described.

#### Example 6

##### *Sequence of the human adenovirus type 35*

Ad35 viruses were propagated on PER.C6 cells and DNA was isolated as described in example 4. The total sequence was generated by Qiagen Sequence Services (Qiagen GmbH, Germany). Total viral DNA was sheared by sonification and the ends of the DNA were made blunt by T4 DNA polymerase. Sheared blunt fragments were size fractionated on agarose gels and gel slices corresponding to DNA fragments of 1.8 to 2.2 kb were obtained. DNA was purified from the gel slices by the QIAquick gel extraction protocol and subcloned into a shotgun library of pUC19 plasmid cloning vectors. An array of clones in 96-wells plates covering the target DNA 8 (+/- 2) times was used to generate the total sequence. Sequencing was performed on Perkin-Elmer 9700 thermo cyclers using BigDyeTerminator chemistry and AmpliTaq FS DNA polymerase followed by purification of sequencing reactions using QIAGEN DyeEx 96 technology. Sequencing reaction products were then subjected to automated separation and detection of fragments on ABI 377 XL 96 lane sequencers. Initial sequence

contig sequence and gaps were filled in by primer walking reads on the target DNA or by direct sequencing of PCR products. The ends of the virus turned out to be absent in the shotgun library, most probably due to cloning difficulties resulting from the amino acids of pTP that remain bound to the ITR sequences after proteinase K digestion of the viral DNA. Additional sequence runs on viral DNA solved most of the sequence in those regions, however it was difficult to obtain a clear sequence of the most terminal nucleotides. At the 5' end the sequence obtained was 5'-CCAATAATATACCT ...-3' while at the 3' end the obtained sequence was 5'-...AGGTATATTATTGATGATGGG-3'. Most human adenoviruses have a terminal sequence 5'-CATCATCAATAATATACC-3'. In addition, a clone representing the 3' end of the Ad35 DNA obtained after cloning the terminal 7 kb Ad35 EcoRI fragment into pBr322 (see example 4) also turned out to have the typical CATCATCAATAAT... sequence. Therefore, Ad35 may have the typical end sequence and the differences obtained in sequencing directly on the viral DNA are due to artefacts correlated with run-off sequence runs and the presence of residual amino acids of pTP. The total sequence of Ad35 with corrected terminal sequences is given in Figure 6. Based sequence homology with Ad5 (genbank # M72360) and Ad7 (partial sequence Genbank # X03000) and on the location of open reading frames, the organization of the virus is identical to the general organization of most human adenoviruses, especially the subgroup B viruses. The total length of the genome is 34794 basepairs.

30

#### Example 7

*Construction of a plasmid-based vector system to generate recombinant Ad35-based viruses.*

A functional plasmid-based vector system to generate recombinant adenoviral vectors comprises the following components:

35

1. An adapter plasmid comprising a left ITR and packaging sequences derived from Ad35 and at least one restriction site for insertion of an heterologous expression cassette and lacking E1 sequences. Furthermore, the adapter  
5 plasmid contains Ad35 sequences 3' from the E1B coding region including the pIX promoter and coding sequences enough to mediate homologous recombination of the adapter plasmid with a second nucleic acid molecule.
2. A second nucleic acid molecule, comprising sequences  
10 homologous to the adapter plasmid, and Ad35 sequences necessary for the replication and packaging of the recombinant virus, that is early, intermediate and late genes that are not present in the packaging cell.
3. A packaging cell providing at least functional E1  
15 proteins capable of complementing the E1 function of Ad35.

Other methods for the generation of recombinant adenoviruses on complementing packaging cells are known in the art and may be applied to Ad35 viruses without departing from the  
20 invention. As an example, the construction of a plasmid based system, as outlined above, is described in detail below.

#### 1) Construction of Ad35 adapter plasmids.

25 Hereto, the adapter plasmid pAdApt (Figure 7; described in example 2) was first modified to obtain adapter plasmids that contain extended polylinkers and that have convenient unique restriction sites flanking the left ITR and the adenovirus sequence at the 3' end to enable liberation of  
30 the adenovirus insert from plasmid vector sequences. Construction of these plasmids is described below in detail: Adapter plasmid pAdApt (Example 2) was digested with SalI and treated with Shrimp Alkaline Phosphatase to reduce religation. A linker, composed of the following two  
35 phosphorylated and annealed oligo's: ExSalPacF 5' - TCG ATG GCA AAC AGC TAT TAT GGG TAT TAT GGG TTC GAA TTA ATT AA- 3';

and ExSalPacR 5' - TCG ATT AAT TAA TTC GAA CCC ATA ATA CCC  
ATA ATA GCT GTT TGC CA- 3'; was directly ligated into the  
digested construct, thereby replacing the SalI restriction  
site by Pi-PspI, SwaI and PacI. This construct was named  
5 pADAPT+ExSalPac linker. Furthermore, part of the left ITR of  
pAdapt was amplified by PCR using the following primers:  
PCLIPMSF: 5'- CCC CAA TTG GTC GAC CAT CAT CAA TAA TAT ACC  
TTA TTT TGG -3' and pCLIPBSRGI: 5'- GCG AAA ATT GTC ACT TCC  
TGT G - 3'. The amplified fragment was digested with MunI  
10 and BsrGI and cloned into pAd5/Clip (see Example 2), which  
was partially digested with EcoRI and after purification  
digested with BsrGI, thereby re-inserting the left ITR and  
packaging signal. After restriction enzyme analysis, the  
construct was digested with ScaI and SgrAI and an 800 bp  
15 fragment was isolated from gel and ligated into ScaI/SgrAI  
digested pADAPT+ExSalPac linker. The resulting construct,  
named pIPspSalAdapt, was digested with SalI,  
dephosphorylated, and ligated to the phosphorylated  
ExSalPacF/ExSalPacR doublestranded linker mentioned above. A  
20 clone in which the PacI site was closest to the ITR was  
identified by restriction analysis and sequences were  
confirmed by sequence analysis. This novel pAdapt construct,  
termed pIPspAdapt (Figure 8) thus harbors two ExSalPac  
linkers containing recognition sequences for PacI, PI-PspI  
25 and BstBI, which surround the adenoviral part of the  
adenoviral adapter construct, and which can be used to  
linearize the plasmid DNA prior to cotransfection with  
adenoviral helper fragments.

30 In order to further increase transgene cloning permutations  
a number of polylinker variants were constructed based on  
pIPspAdapt. For this purpose pIPspAdapt was first digested  
with EcoRI and dephosphorylated. A linker composed of the  
following two phosphorylated and annealed oligo's:  
35 Ecolinker+: 5' -AAT TCG GCG CGC CGT CGA CGA TAT CGA TAG CGG  
CCG C -3' and Ecolinker-: 5' -AAT TGC GGC CGC TAT CGA TAT

CGT CGA CGG CGC GCC G -3' was ligated into this construct, thereby creating restriction sites for AscI, SalI, EcoRV, ClaI and NotI. Both orientations of this linker were obtained and sequences were confirmed by restriction

5 analysis and sequence analysis. The plasmid containing the polylinker in the order 5' HindIII, KpnI, AgeI, EcoRI, AscI, SalI, EcoRV, ClaI, NotI, NheI, HpaI, BamHI and XbaI was termed pIPspAdapt1 (Figure 9) while the plasmid containing the polylinker in the order HindIII, KpnI, AgeI, NotI, ClaI,  
10 EcoRV, SalI, AscI, EcoRI, NheI, HpaI, BamHI and XbaI was termed pIPspAdapt2.

To facilitate the cloning of other sense or antisense constructs, a linker composed of the following two oligonucleotides was designed, to reverse the polylinker of  
15 pIPspAdapt: HindXba+ 5'-AGC TCT AGA GGA TCC GTT AAC GCT AGC GAA TTC ACC GGT ACC AAG CTT A-3'; HindXba- 5'-CTA GTA AGC TTG GTA CCG GTG AAT TCG CTA GCG TTA ACG GAT CCT CTA G-3'. This linker was ligated into HindIII/XbaI digested pIPspAdapt and the correct construct was isolated.

20 Confirmation was done by restriction enzyme analysis and sequencing. This new construct, pIPspAdaptA, was digested with EcoRI and the above mentioned Ecolinker was ligated into this construct. Both orientations of this linker were obtained, resulting in pIPspAdapt3 (Figure 10), which  
25 contains the polylinker in the order XbaI, BamHI, HpaI, NheI, EcoRI, AscI, SalI, EcoRV, ClaI, NotI, AgeI, KpnI and HindIII. All sequences were confirmed by restriction enzyme analysis and sequencing.

30 Adapter plasmids based on Ad35 were then constructed as follows:

The left ITR and packaging sequence corresponding to Ad35 wt sequences nucleotides 1 to 464 (Figure 6) were amplified by PCR on wtAd35 DNA using the following primers:

35 Primer 35F1:

5'-CGG AAT TCT TAA TTA ATC GAC ATC ATC AAT AAT ATA CCT TAT  
AG-3'

Primer 35R2:

5'-GGT GGT CCT AGG CTG ACA CCT ACG TAA AAA CAG-3'

- 5 Amplification introduces a PacI site at the 5' end and an  
AvrII site at the 3' end of the sequence.

- For the amplification Platinum Pfx DNA polymerase enzyme  
(LTI) was used according to manufacturers instructions but  
with primers at 0.6  $\mu$ M and with DMSO added to a final  
10 concentration of 3%. Amplification program was as follows: 2  
min. at 94°C, (30 sec. 94°C, 30 sec. at 56°C, 1 min. at  
68°C) for 30 cycles, followed by 10 min. at 68°C.

- The PCR product was purified using a pcr purification kit  
(LTI) according to the manufacturers instructions and  
15 digested with PacI and AvrII. The digested fragment was then  
purified from gel using the geneclean kit (Bio 101, Inc.).  
The Ad5-based adapter plasmid pIPspAdApt-3 (figure 10) was  
digested with AvrII and then partially with PacI and the  
5762 bp fragment was isolated in an LMP agarose gel slice  
20 and ligated with the abovementioned PCR fragment digested  
with the same enzymes and transformed into electrocompetent  
DH10B cells (LTI). The resulting clone is named pIPspAdApt3-  
Ad35lITR.

- In parallel, a second piece of Ad35 DNA was amplified using  
25 the following primers:

35F3: 5'- TGG TGG AGA TCT GGT GAG TAT TGG GAA AAC-3'

35R4: 5'- CGG AAT TCT TAA TTA AGG GAA ATG CAA ATC TGT GAG G-  
3'

- The sequence of this fragment corresponds to nucl. 3401 to  
30 4669 of wtAd35 (Figure 6) and contains 1.3 kb of sequences  
starting directly 3' from the E1B 55k coding sequence.

- Amplification and purification was done as described above  
for the fragment containing the left ITR and packaging  
sequence. The PCR fragment was then digested with PacI and  
35 subcloned into pNEB193 vector (New England Biolabs) digested  
with SmaI and PacI. The integrity of the sequence of the



resulting clone was checked by sequence analysis.  
pNEB/Ad35pF3R4 was then digested with BglII and PacI and the Ad35 insert was isolated from gel using the QIAExII kit (Qiagen). pIPspAdApt3-Ad35lITR was digested with BglII and then partially with PacI. The 3624 bp fragment (containing vector sequences, the Ad35 ITR and packaging sequences as well as the CMV promoter, multiple cloning region and polyA signal), was also isolated using the QIAExII kit (Qiagen). Both fragments were ligated and transformed into competent DH10B cells (LTI). The resulting clone, pAdApt35IP3 (Figure 11), has the expression cassette from pIPspAdApt3 but contains the Ad35 left ITR and packaging sequences and a second fragment corresponding to nucl. 3401 to 4669 from Ad35. A second version of the Ad35 adapter plasmid having the multiple cloning site in the opposite orientation was made as follows:  
pIPspAdapt1 (Figure 9) was digested with NdeI and BglII and the 0.7 kbp band containing part of the CMV promoter, the MCS and SV40 polyA was isolated and inserted in the corresponding sites of pAdApt35IP3 generating pAdApt35IP1 (Figure 12).  
pAdApt35.LacZ and pAdApt35.Luc adapter plasmids were then generated by inserting the transgenes from pcDNA.LacZ (digested with KpnI and BamHI) and pAdApt.Luc (digested with HindIII and BamHI) into the corresponding sites in pAdApt35IP1. The generation of pcDNA.LacZ and pAdApt.Luc is described in WO99/55132.

## 2) Construction of cosmid pWE.Ad35.pXI-rITR

Figure 13 presents the various steps undertaken to construct the cosmid clone containing Ad35 sequences from bp 3401 to 34794 (end of the right ITR) that are described in detail below.

A first PCR fragment (pIX-NdeI) was generated using the following primer set:

35F5: 5'-CGG AAT TCG CGG CCG CGG TGA GTA TTG GGA AAA C -3'

35R6: 5'-CGC CAG ATC GTC TAC AGA ACA G-3'

DNA polymerase Pwo (Roche) was used according to manufacturers instructions, however, with an endconcentration of 0.6  $\mu$ M of both primers and using 50 ngr wt Ad35 DNA as template.

- 5 Amplification was done as follows: 2 min. at 94 °C, 30 cycles of 30 sec. at 94 °C, 30 sec. at 65 °C and 1 min. 45 sec. at 72 °C, followed by 8 min. at 68 °C. To enable cloning in the TA cloning vector PCR2.1, a last incubation with 1 unit superTaq polymerase (HT Biotechnology LTD) for  
10 10 min. at 72 °C was performed.

The 3370 bp amplified fragment contains Ad35 sequences from bp 3401 to 6772 with a NotI site added to the 5' end.

Fragments were purified using the PCR purification kit (LTI).

- 15 A second PCR fragment (NdeI-rITR) was generated using the following primers:

35F7: 5'-GAA TGC TGG CTT CAG TTG TAA TC -3'

35R8: 5'- CGG AAT TCG CGG CCG CAT TTA AAT CAT CAT CAA TAA  
TAT ACC-3'

- 20 Amplification was done with pfx DNA polymerase (LTI) according to manufacturer's instructions but with 0.6  $\mu$ M of both primers and 3% DMSO using 10 ngr. of wtAd35 DNA as template. The program was as follows:

- 3 min. at 94 °C and 5 cycles of 30 sec. at 94 °C, 45 sec. at  
25 40 °C, 2 min.45 sec. at 68 °C followed by 25 cycles of 30 sec. at 94 °C, 30 sec. at 60 °C, 2 min.45 sec. at 68 °C. To enable cloning in the TA-cloning vector PCR2.1, a last incubation with 1 unit superTaq polymerase for 10 min. at 72 °C was performed. The 1.6 kb amplified fragment ranging from  
30 nucl. 33178 to the end of the right ITR of Ad35, was purified using the PCR purification kit ( LTI).

- Both purified PCR fragments were ligated into the PCR2.1 vector of the TA-cloning kit (Invitrogen) and transformed into STBL-2 competent cells (LTI). Clones containing the  
35 expected insert were sequenced to confirm correct amplification. Next, both fragments were excised from the

vector by digestion with NotI and NdeI and purified from gel using the geneclean kit (BIO 101, Inc.). Cosmid vector pWE15 (Clontech) was digested with NotI, dephosphorylated and also purified from gel. These three fragments were ligated and  
5 transformed into STBL2 competent cells (LTI). One of the correct clones that contained both PCR fragments was then digested with NdeI and the linear fragment was purified from gel using the geneclean kit. Ad35 wtDNA was digested with NdeI and the 26.6 kb fragment was purified from LMP gel  
10 using agarase enzym (Roche) according to the manufacturers instructions. These fragments were ligated together and packaged using  $\lambda$  phage packaging extracts (Stratagene) according to the manufacturer's protocol. After infection into STBL-2 cells, colonies were grown on plates and  
15 analyzed for presence of the complete insert. One clone with the large fragment inserted in the correct orientation and having the correct restriction patterns after independent digestions with three enzymes (NcoI, PvuII and ScaI) was selected. This clone is named pWE.Ad35.pIX-rITR. It contains  
20 the Ad35 sequences from bp 3401 to the end and is flanked by NotI sites (Figure 14).

### 3) Generation of Ad35 based recombinant viruses on PER.C6.

Wild type Ad35 virus can be grown on PER.C6 packaging  
25 cells to very high titers. However, whether the Ad5-E1 region that is present in PER.C6 is able to complement E1-deleted Ad35 recombinant viruses is unknown. To test this, PER.C6 cells were cotransfected with the above described adapter plasmid pAdApt35.LacZ and the large backbone  
30 fragment pWE.Ad35.pIX-rITR. First, pAdApt35.LacZ was digested with PacI and pWE.Ad35.pIX-rITR was digested with NotI. Without further purification 4  $\mu$ gr of each construct was mixed with DMEM (LTI) and transfected into PER.C6 cells, seeded at a density of  $5 \times 10^6$  cells in a T25 flask the day  
35 before, using Lipofectamin (LTI) according to the

manufacturers instructions. As a positive control, 6 $\mu$ gr of PacI digested pWE.Ad35.pIX-rITR DNA was cotransfected with a 6.7 kb NheI fragment isolated from Ad35 wt DNA containing the left end of the viral genome including the E1 region.

5 The next day medium (DMEM with 10% FBS and 10mM MgCl<sub>2</sub>) was refreshed and cells were further incubated. At day 2 following the transfection, cells were trypsinized and transferred to T80 flasks. The positive control flask showed CPE at five days following the transfection, showing that

10 the pWE.Ad35.pIX-rITR construct is functional at least in the presence of Ad35-E1 proteins. The transfection with the Ad35 LacZ adapter plasmid and pWE.Ad35.pIX-rITR did not give rise to CPE. These cells were harvested in the medium at day 10 and freeze/thawed once to release virus from the cells. 4

15 ml of the harvested material was added to a T80 flask with PER.C6 cells (at 80% confluency) and incubated for another five days. This harvest/re-infection was repeated for two times but there was no evidence for virus associated CPE. From this experiment it seems that the Ad5-E1 proteins are

20 not, or not well enough, capable of complementing Ad35 recombinant viruses, however, it may be that the sequence overlap of the adapter plasmid and the pWE.Ad35.pIX-rITR backbone plasmid is not large enough to efficiently recombine and give rise to a recombinant virus genome. The

25 positive control transfection was done with a 6.7 kb left end fragment and therefore the sequence overlap was about 3.5 kb. The adapter plasmid and the pWE.Ad35.pIX-rITR fragment have a sequence overlap of 1.3 kb. To check whether the sequence overlap of 1.3 kb is too small for efficient

30 homologous recombination, a cotransfection was done with PacI digested pWE.Ad35.pIX-rITR and a PCR fragment of Ad35 wtDNA generated with the above mentioned 35F1 and 35R4 using the same procedures as described before. The PCR fragment thus contains left end sequences up to bp 4669 and therefore

35 has the same overlap sequences with pWE.Ad35.pIX-rITR as the adapter plasmid pAdApt35.LacZ but has Ad35 E1 sequences.

Following PCR column purification, the DNA was digested with SalI to remove possible intact template sequences. A transfection with the digested PCR product alone served as a negative control. Four days after the transfection, CPE  
5 occurred in the cells transfected with the PCR product and the Ad35 pIX-rITR fragment, and not in the negative control. This shows that 1.3 kb overlapping sequences is sufficient to generate viruses in the presence of Ad35 E1 proteins. From these experiments we conclude that the presence of at  
10 least one of the Ad35.E1 proteins is necessary to generate recombinant Ad35 based vectors from plasmid DNA on Ad5 complementing cell lines.

#### Example 8

##### 15 1) Construction of Ad35.E1 expression plasmids

Since Ad5-E1 proteins in PER.C6 are not capable of complementing Ad35 recombinant viruses efficiently, Ad35 E1 proteins have to be expressed in Ad5 complementing cells (e.g. PER.C6) or a new packaging cell line expressing Ad35  
20 E1 proteins has to be made, starting from either diploid primary human cells or established cell lines not expressing adenovirus E1 proteins. To address the first possibility, the Ad35 E1 region was cloned in expression plasmids as described below.

25 First, the Ad35 E1 region from bp 468 to bp 3400 was amplified from wtAd35 DNA using the following primer set:  
35F11: 5'-GGG GTA CCG AAT TCT CGC TAG GGT ATT TAT ACC-3'  
35F10: 5'-GCT CTA GAC CTG CAG GTT AGT CAG TTT CTT CTC CAC TG-3'

30 This PCR introduces a KpnI and EcoRI site at the 5' end and a SbfI and XbaI site at the 3' end.

Amplification on 5 ngr. template DNA was done with Pwo DNA polymerase (Roche) using manufacturers instructions, however, with both primers at a final concentration of 0.6  
35  $\mu$ M. The program was as follows: 2 min. at 94 °C, 5 cycles of 30 sec. at 94 °C, 30 sec. at 56 °C and 2 min. at 72 °C,

followed by 25 cycles of 30 sec. at 94°C, 30 sec. at 60 °C and 2 min. at 72 °C, followed by 10 min. at 72 °C. PCR product was purified by a PCR purification kit (LTI) and digested with KpnI and XbaI. The digested PCR fragment was then ligated to the expression vector pRSVhvbvNeo (see below), also digested with KpnI and XbaI. Ligations were transformed into competent STBL-2 cells (LTI) according to manufacturers instructions and colonies were analysed for the correct insertion of Ad35E1 sequences into the polylinker in between the RSV promoter and HBV polyA. The resulting clone was named pRSV.Ad35-E1 (Figure 15). The Ad35 sequences in pRSV.Ad35-E1 were checked by sequence analysis.

pRSVhvbvNeo was generated as follows: pRc-RSV (Invitrogen) was digested with PvuII, dephosphorylated with TSAP enzyme (LTI) and the 3 kb vector fragment was isolated in low melting point agarose (LMP). Plasmid pPGKneopA (Figure 16; described in WO96/35798, was digested with SspI completely to linearise the plasmid and facilitate partial digestion with PvuII. Following the partial digestion with PvuII, the resulting fragments were separated on a LMP agarose gel and the 2245 bp PvuII fragment, containing the PGK promoter, neomycine resistance gene and HBVpolyA, was isolated. Both isolated fragments were ligated to give the expression vector pRSV-pNeo that now has the original SV40prom-neo-SV40polyA expression cassette replaced by a PGKprom-neo-HBVpolyA cassette (Figure 17). This plasmid was further modified to replace the BGHpA with the HBVpA as follows: pRSVpNeo was linearised with ScaI and further digested with XbaI. The 1145 bp fragment, containing part of the Amp gene and the RSV promoter sequences and polylinker sequence, was isolated from gel using the GeneClean kit (Bio Inc. 101). Next pRSVpNeo was linearised with ScaI and further digested with EcoRI partially and the 3704 bp fragment containing the PGKneo cassette and the vector sequences were isolated from gel as above. A third fragment, containing the HBV polyA

sequence flanked by XbaI and EcoRI at the 5' and 3' end respectively, was then generated by PCR amplification on pRSVpNeo using the following primer set:

HBV-F: 5'- GGC TCT AGA GAT CCT TCG CGG GAC GTC -3' and

5 HBV-R: 5'- GGC GAA TTC ACT GCC TTC CAC CAA GC -3'.

Amplification was done with Elongase enzyme (LTI) according to the manufacturers instructions with the following conditions: 30 seconds at 94°C, then 5 cycles of 45 seconds at 94 °C, 1 minute at 42 °C and 1 minute 68 °C, followed by 10 30 cycles of 45 seconds at 94 °C, 1 minute at 65 °C and 1 minute at 68 °C, followed by 10 minutes at 68 °C. The 625 bp PCR fragment was then purified using the Qiaquick PCR purification kit, digested with EcoRI and XbaI and purified from gel using the Geneclean kit. The three isolated 15 fragments were ligated and transformed into DH5α competent cells (LTI) to give the construct pRSVhbmNeo (Figure 18). In this construct the transcription regulatory regions of the RSV expression cassette and the neomycine selection marker are modified to reduce overlap with adenoviral vectors that 20 often contain CMV and SV40 transcription regulatory sequences.

2) Generation of Ad35 recombinant viruses on PER.C6 cells cotransfected with an Ad35-E1 expression construct.

25 PER.C6 cells were seeded at a density of  $5 \times 10^6$  cells in a T25 flask and the next day transfected with a DNA mixture containing:

- 1 µg pAdApt35.LacZ digested with PacI
- 5 µg pRSV.Ad35E1 undigested
- 30 - 2 µg pWE.Ad35.pIX-rITR digested with NotI

Transfection was done using Lipofectamine according to the manufacturers instructions. Five hours after addition of the transfection mixture to the cells, medium was removed and replaced by fresh medium. After two days cells were 35 transferred to T80 flasks and further cultured. One week post-transfection 1 ml of the medium was added to A549 cells

and the following day cells were stained for LacZ expression. Blue cells were clearly visible after two hours of staining indicating that recombinant LacZ expressing viruses were produced. The cells were further cultured but  
5 no clear appearance of CPE was noted. However, after 12 days clumps of cells appeared in the monolayer and 18 days following transfection cells were detached. Cells and medium were then harvested, freeze-thawed once and 1 ml of the crude lysate was used to infect PER.C6 cells in a 6-well  
10 plate. Two days after infection cells were stained for LacZ activity. After two hours 15% of the cells were stained blue. To test for the presence of wt and / or replicating competent viruses, A549 cells were infected with these  
15 viruses and further cultured. No signs of CPE were found indicating the absence of replication competent viruses. These experiments show that recombinant AdApt35.LacZ viruses were made on PER.C6 cells cotransfected with an Ad35-E1 expression construct.

20 3) Ad35 recombinant viruses escape neutralization in human serum containing neutralizing activity to Ad5 viruses.

The AdApt35.LacZ viruses were then used to investigate infection in the presence of serum that contains neutralizing activity to Ad5 viruses. Purified Ad5-based  
25 LacZ virus served as a positive control for NA. Hereto, PER.C6 cells were seeded in a 24-wells plate at a density of  $2 \times 10^5$  cells/well. The next day a human serum sample with high neutralizing activity to Ad5 was diluted in culture medium in five steps of five times dilutions. 0.5 ml of  
30 diluted serum was then mixed with  $4 \times 10^6$  virus particles AdApt5.LacZ virus in 0.5 ml medium and after 30 minutes of incubation at 37 °C, 0.5 ml of the mixture was added to PER.C6 cells in duplicate. For the AdApt35.LacZ viruses, 0.5 ml of the diluted serum samples were mixed with 0.5 ml crude  
35 lysate containing AdApt35.LacZ virus and after incubation 0.5 ml of this mixture was added to PER.C6 cells in duplo.



Virus samples incubated in medium without serum was used as a positive control for infection. After two hours of infection at 37 °C, medium was added to reach a final volume of 1 ml and cells were further incubated. Two days after infection cells were stained for LacZ activity. The results are shown in Table II. From these results it is clear that whereas AdApt5.LacZ viruses are efficiently neutralized, AdApt35.LacZ viruses remain infectious irrespective of the presence of human serum. This proves that recombinant Ad35-based viruses escape neutralization in human sera that contain NA to Ad5-based viruses.

#### Example 9:

*An Ad5/fiber35 chimeric vector with cell type specificity for hemopoietic CD34<sup>+</sup>Lin<sup>-</sup> stem cells*

In example 3 we have described the generation of a library of Ad5 based adenoviruses harboring fiber proteins of other serotypes. As a non-limiting example for the use of this library we here describe the identification of fiber-modified adenoviruses that show improved infection of hemopoietic stem cells.

Cells isolated from human bone marrow, umbilical cord blood, or mobilized peripheral blood carrying the flow cytometric phenotype of being positive for the CD34 antigen and negative for the early differentiation markers CD33, CD38, and CD71 (lin<sup>-</sup>) are commonly referred to as hemopoietic stem cells (HSC). Genetic modification of these cells is of major interest since all hemopoietic lineages are derived from these cells and therefore the HSC is a target cell for the treatment of many acquired or congenital human hemopoietic disorders. Examples of diseases that are amenable for genetic modification of HSC, but not limited to, include Hurlers disease, Hunters disease, Sanfilippo disease, Morquio disease, Gaucher disease, Farbers disease, Niemann-

Pick disease, Krabbe disease, Metachromatic Leucodystrophy, I-cell disease, severe immunodeficiency syndrome, Jak-3 deficiency, Fucosidose deficiency, thalassemia, and erythropoietic porphyria. Besides these hemopoietic disorders also strategies to prevent or treat acquired immunodeficiency syndrome (AIDS) and hemopoietic cancers are based on the genetic modification of HSCs or cells derived from the HSCs such as CD4 positive T lymphocytes in case of AIDS. The examples listed above thus aim at introducing DNA into the HSC in order to complement on a genetic level for a gene and protein deficiency. In case of strategies for AIDS or cancer, the DNA to be introduced into the HSC can be anti-viral genes or suicide genes.

Besides the examples listed above, there are several other areas in which efficient transduction of HSCs using adenoviral vectors plays an important role. For instance in the field of tissue engineering. In this area it is important to drive differentiation of HSCs to specific lineages. Some, non-limiting, examples are ex vivo bone formation, cartilage formation, skin formation, as well as the generation of T-cell precursors or endothelial cell precursors. The generation of bone, cartilage or skin in bioreactors can be used for transplantation after bone fractures or spinal cord lesions or severe burn injuries. Naturally, transduced cells can also directly be re-infused into a patient. The formation of large numbers of endothelial cell precursor from HSCs is of interest since these endothelial precursor cells can home, after re-infusion, to sites of cardiovascular injury such as ischemia. Likewise, the formation of large numbers of T-cells from HSCs is of interest since these T-cell precursors can be primed, ex vivo, to eradicate certain targets in the human body after reinfusion of the primed T-cells. Preferred targets in the human body can be tumours or virus infected cells.

From the examples described above, it can be concluded that efficient gene delivery to HSCs is a major interest for the field of gene therapy. Therefore, alteration of the adenovirus serotype 5 host cell range to be able to target HSCs *in vitro* as well as *in vivo* is a major interest of the invention. To identify a chimeric adenovirus with preferred infection characteristics for human HSCs, we generated a library of Ad5 based viruses carrying the fiber molecule from alternative serotypes (serotypes 8, 9, 13, 16, 17, 32, 35, 45, 40-L, 51). The generation of this fiber modified library is described in example 3. Ad5 was taken along as a reference. A small panel of this library was tested on human TF-1 (erythroid leukemia, ATCC CRL-2003) whereas all chimaeric viruses generated were tested on human primary stroma cells and human HSCs. Human TF-1 cell were routinely maintained in DMEM supplemented with 10% FCS and 50 ng/ ml IL-3 (Sandoz, Basel, Switzerland). Human primary fibroblast-like stroma, isolated from a bone marrow aspirate, is routinely maintained in DMEM/ 10% FCS. Stroma was seeded at a concentration of  $1 \times 10^5$  cells per well of 24-well plates. 24 hours after seeding cells were exposed for 2 hours to 1000 virus particles per cell of Ad5, Ad5.Fib16, Ad5.Fib17, Ad5.Fib35, Ad5.Fib40-L, or Ad5.Fib51 all carrying the green fluorescent protein (GFP) as a marker. After 2 hours cells were washed with PBS and reseeded in medium without addition of virus. TF-1 cells were seeded at a concentration of  $2 \times 10^5$  cells per well of 24-well plates and were also exposed for 2 hours to 1000 virus particles of the different chimeric adenoviruses. Virus was removed by washing the cells after the 2 hours exposure. Both cell types were harvested 48 hours after virus exposure and analysed for GFP expression using a flow cytometer. The results on TF-1 cells, shown in figure 19, demonstrates that chimeric adenoviruses carrying a fiber from serotypes 16, 35, or 51 (all derived from adenovirus subgroup B) have preferred infection characteristics as compared to Ad5 (subgroup C), Ad5.Fib17

(subgroup D); or Ad5.Fib40-L (subgroup F). Primary human stroma was tested since these cells are commonly used as a "feeder" cell to allow proliferation and maintenance of HSCs under ex vivo culture conditions. In contrast to the

5 transduction of TF-1 cells, none of the fiber chimeric adenoviruses were able to efficiently transduce human primary stroma (Figure 20). Reasonable infection of human fibroblast-like primary stroma was observed only with Ad5 despite the observation that none of the known receptor

10 molecules are expressed on these cells (see table III). The absence of infection of human stroma using the chimeric viruses is advantageous since in a co-culture setting, the chimeric adenovirus will not be absorbed primarily by the stroma "feeder" cells.

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To test the transduction capacity of the fiber chimaeric viruses, a pool of umbilical cord blood (3 individuals) was used for the isolation of stem cells. CD34<sup>+</sup> cells were isolated from mononuclear cell preparation using a MACS

20 laboratory separation system (Miltenyi Biotec) using the protocol supplied by the manufacturer. Of the CD34<sup>+</sup> cells,  $2 \times 10^5$  were seeded in a volume of 150  $\mu$ l DMEM (no serum; Gibco, Gaithersburg, MD) and 10  $\mu$ l of chimeric adenovirus (to give a final virus particles/cell ratio of 1000) was added.

25 The chimeric adenoviruses tested were Ad5, Ad5.Fib16, Ad5.Fib35, Ad5Fib17, Ad5.Fib51 all containing Green fluorescent protein (GFP) as a marker. Cells were incubated for 2 hours in a humidified atmosphere of 10% CO<sub>2</sub> at 37°C. Thereafter, cells were washed once with 500  $\mu$ l DMEM and

30 resuspended in 500  $\mu$ l of StemPro-34 SF medium (Life Technologies, Grand Island, NY). Cells were then cultured for 5 days in 24-well plates (Greiner, Frickenhausen, Germany) on irradiated (20 Gy) pre-established human bone marrow stroma (ref 1), in a

35 humidified atmosphere of 10% CO<sub>2</sub> at 37°C. After 5 days, the entire cell population was collected by trypsinization with

100  $\mu$ l 0.25% Trypsin-EDTA (Gibco). The number of cells before and after 5 days of culture was determined using a hemacytometer. The number of CD34<sup>+</sup> and CD34<sup>+</sup>CD33,38,71<sup>-</sup> cells in each sample was calculated from the total number of cells recovered and the frequency of the CD34<sup>+</sup>CD33,38,71<sup>-</sup> cells in the whole population as determined by FACS analysis. The transduction efficiency was determined by FACS analysis while monitoring in distinct sub populations the frequency of GFP expressing cells as well as the intensity of GFP per individual cell. The results of this experiment, shown in figure 21, demonstrates that adenovirus serotype 5 or the chimeric adenovirus Ad5.Fib17 does not infect CD34<sup>+</sup>Lin<sup>-</sup> cells as witnessed by the absence of GFP expression. In contrast, with the chimeric viruses carrying the fiber molecule of serotypes 16, 51, or 35 high percentages of GFP positive cells are scored in this cell population. Specificity for CD34<sup>+</sup>Lin<sup>-</sup> is demonstrated since little GFP expression is observed in CD34<sup>+</sup> cells that are also expressing CD33, CD38, and CD71. Subfractioning of the CD34<sup>+</sup>Lin<sup>-</sup> cells (Figure 22) showed that the percentage of cells positive for GFP declines using Ad5.Fib16, Ad5.Fib35, or Ad5.Fib51 when the cells become more and more positive for the early differentiation markers CD33 (myeloid), CD71 (erythroid), and CD38 (common early differentiation marker). These results thus demonstrate the specificity of the chimeric adenoviruses Ad5.Fib16, Ad5.Fib35, and Ad5.Fib51 for HSCs. Figure 23 shows an alignment of the Ad5 fiber with the chimeric B-group fiber proteins derived from Ad16, 35 and 51. By determining the number of cells recovered after the transduction procedure the toxicity of adenovirus can be determined. The recovery of the amount of CD34<sup>+</sup> cells as well as the amount of CD34<sup>+</sup>Lin<sup>-</sup> (Figure 24) demonstrates that a 2 hour exposure to 1000 adenovirus particles did not have an effect on the number of cells recovered.

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**Example 10**

*An Ad5/fiber35 chimeric vector with cell type specificity for Dendritic cells*

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Dendritic cells are antigen presenting cells (APC), specialized to initiate a primary immune response and able to boost a memory type of immune response. Dependent on their stage of development, DC display different functions: immature DC are very efficient in the uptake and processing of antigens for presentation by Major Histocompatibility Complex (MHC) class I and class II molecules; whereas mature DC, being less effective in antigen capture and processing, perform much better at stimulating naive and memory CD4<sup>+</sup> and CD8<sup>+</sup> T cells, due to the high expression of MHC molecules and co-stimulatory molecules at their cell surface. The immature DCs mature *in vivo* after uptake of antigen, travel to the T-cell areas in the lymphoid organs, and prime T-cell activation.

Since DCs are the cells responsible for triggering an immune response there has been a long standing interest in loading DCs with immunostimulatory proteins, peptides or the genes encoding these proteins to trigger the immune system. The applications for this strategy are in the field of cancer treatment as well as in the field of vaccination. So far, anti-cancer strategies have focussed primarily on *ex vivo* loading of DCs with antigen (protein or peptide). These studies have revealed that this procedure resulted in induction of cytotoxic T cell activity. The antigens used to load the cells are generally identified as being tumor specific. Some, non-limiting, examples of such antigens are GP100, mage, or Mart-1 for melanoma.

Besides treatment of cancer many other potential human diseases are currently being prevented through vaccination. In the vaccination strategy, a "crippled" pathogen is

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presented to the immune system via the action of the antigen presenting cells, i.e. the immature DCs. Well-known examples of disease prevention via vaccination strategies include Hepatitis A,B, and C, influenza, rabies, yellow fever, measles. Besides these well-known vaccination programs, research programs for treatment of malaria, ebola, river blindness, HIV and many other diseases are being developed. Many of the above mentioned pathogens are considered to dangerous for the generation of a "crippled" pathogen vaccine. This latter thus calls for the isolation and characterization of proteins of each pathogen which is able to mount a "full blown" immune response thus resulting in complete protection upon challenge with wild type pathogen. For this strategy of loading DCs with immunostimulatory proteins or peptides to become therapeutically feasible At least two distinct criteria have to be met 1) the isolation of large numbers of DCs which can be isolated, manipulated, and reinfused into a patient, making the procedure autologous. To date, it is possible to obtain such large quantities of immature DCs from cultured peripheral blood monocytes from any given donor. 2) a vector which can transduce DCs efficiently such that the DNA encoding for an immunostimulatory protein can be delivered. The latter is extremely important since it has become clear that the time required for DCs to travel to the lymphoid organs is such that most proteins or peptides are already released from the DCs resulting in incomplete immune priming. Because DCs are terminally differentiated and thus non-dividing cells, recombinant adenoviral vectors are are being considered for delivering the DNA encoding for antigens to DCs. Ideally this adenovirus should have a high affinity for dendritic cells but also should not be recognized by neutralizing antibodies of the host such that *in vivo* transduction of DCs can be accomplished. This latter would omit the need for *ex vivo* manipulations of DCs but would result in a medical

procedure identical to the vaccination programs which are currently in place, i.e. intramuscular or subcutaneous injection predominantly. Thus, DC transduced by adenoviral vectors encoding an immunogenic protein may be ideally suited to serve as natural adjuvants for immunotherapy and vaccination

From the above described examples, it can be concluded that efficient gene delivery to DCs is a major interest for the field of gene therapy. Therefore, alteration of the adenovirus serotype 5 host cell range to be able to target DCs *in vitro* as well as *in vivo* is a major interest of the invention. To identify a chimeric adenovirus with preferred infection characteristics for human DCs, we generated a library of Ad5 based viruses carrying the fiber molecule from alternative serotypes (serotypes 8, 9, 13, 16, 17, 32, 35, 45, 40-L, 51). Ad5 was taken along as a reference. We evaluated the susceptibility of human monocyte derived immature and mature DC to recombinant chimeric adenoviruses expressing different fibers.

Human PBMC from healthy donors were isolated through Ficoll-Hypaque density centrifugation. Monocytes were isolated from PBMC by enrichment for CD14<sup>+</sup> cells using staining with FITC labeled anti-human CD 14 monoclonal antibody (Becton Dickinson), anti FITC microbeads and MACS separation columns (Miltenyi Biotec).

This procedure usually results in a population of cells that are < 90 % CD14<sup>+</sup> as analysed by FACS. Cells were placed in culture using RPMI-1640 medium (Gibco) containing 10% Foetal Bovine Serum (Gibco), 200 ng/ml rhu GM-CSF (R&D/ITK diagnostics, 100 ng/ml rhu IL-4 (R&D/ITK diagnostics) and cultured for 7 days with feeding of the cultures with fresh medium containing cytokines on alternate days. The immature DC resulting from this procedure after 7 days express a phenotype CD83<sup>-</sup>, CD14<sup>low</sup> or CD14<sup>-</sup>, HLA-DR<sup>+</sup>, as was demonstrated by FACS analysis. Immature DC are matured by culturing the



cells in medium containing 100 ng/ml TNF- $\alpha$  for 3 days, where after they expressed CD83 on their cell surface.

In a pilot experiment  $5 \cdot 10^5$  immature DCs were seeded in wells of 24-well plates and exposed for 24 hours to 100 and  
5 1000 virus particles per cell of each fiber recombinant virus. Virus tested was adenovirus serotype 5 (Ad5), and the fiber chimeric viruses based on Ad5: Ad5.Fib12, Ad5.Fib16, Ad5.Fib28, Ad5.Fib32, Ad5.Fib40-L (long fiber of serotype 40), Ad5.Fib49, and Ad5.Fib51 (where Fibxx stands for the  
10 serotype of which the fiber molecule is derived). these viruses are derived from subgroup C, A, B, D, D, F, D, and B respectively. After 24-hours cells were lysed (1% Triton X-100/ PBS) and luciferase activity was determined using a protocol supplied by the manufacturer (Promega, Madison, WI,  
15 USA). The results of this experiment, shown in figure 25, demonstrates that Ad5 poorly infects immature DCs as witnessed by the low level of transgene expression. In contrast, Ad5.Fib16 and Ad5.Fib51 (both a B-group fiber chimeric virus) and also Ad5.Fib40-L (Subgroup F) show  
20 efficient infection of immature DCs based on luciferase transgene expression.

In a second experiment,  $5 \cdot 10^5$  immature and mature DC were infected with 10000 virus particles per cell of Ad5, Ad5.Fib16, Ad5.Fib40-L, and Ad5.Fib51 all carrying the LacZ  
25 gene as a marker. LacZ expression was monitored by flow cytometric analysis using a CM-FDG kit system and the instructions supplied by the manufacturer (Molecular probes, Leiden, The Netherlands). The results of this experiment, shown in figure 26, correlates with the previous experiment  
30 in that Ad5.Fib16 and Ad5.Fib51 are superior to Ad5 in transducing mature and immature human DCs. Also, this experiment shows that Ad5.Fib40-L is not as good as Ad5.Fib16 and Ad5.Fib51 but better than Ad5.

Based on these results we tested other chimeric adenoviruses  
35 containing fibers of B group viruses e.g. Ad5.Fib11 and Ad5.Fib35 for there capacity to infect DCs. We focussed on

immature DCs since these are the cells that process an expressed transgene product into MHC class I and II presentable peptides. Immature DC's were seeded at a cell density of  $5 \cdot 10^5$  cells/well in 24 well plates (Costar) and

5 infected with 1000 and 5000 virus particles per cell after which the cells were cultured for 48 hours under conditions for immature DCs prior to cell lysis and Luciferase activity measurements. The result of this experiment, shown in figure

10 27, demonstrate that Ad5 based chimeric adenoviruses containing fibers of group-B viruses efficiently infect immature DCs. In a fourth experiment we again infected immature DCs identically as described in the former experiments but this time Ad5, Ad5.Fib16, and Ad5.Fib35 were used carrying green fluorescent protein (GFP) as a

15 markergene. The results on GFP expression measured with a flow cytometer 48 hours after virus exposure is shown in figure 28 and correlates with the data obtained so far. Thus, the results so far are consistent in that Ad5 based vectors carrying a fiber from a alternative adenovirus

20 derived from subgroup B predominantly fiber of 35, 51, 16, and 11 are superior to Ad5 for transducing human DCs. The adenoviruses disclosed herein are also very suitable in vaccination of animals. To illustrate this, we tested DCs derived from mouse and chimpanzee to identify whether these

25 viruses can be used in these animal models. This latter in particular since the receptor for human adenovirus derived from subgroup B is unknown to date and therefore it is unknown whether this protein is conserved among species. For both species immature DCs were seeded at a density of  $10^5$

30 cells per well of 24-well plates. Cells were subsequently exposed for 48 hours to 1000 virus particles per cell of Ad5, Ad5Fib16, and Ad5.Fib51 in case of mouse DC and Ad5, and Ad.Fib35 in case of chimpanzee DCs (see figure 29). The mouse experiment was performed with viruses carrying

35 luciferase as a marker and demonstrated approximately 10-50 fold increased luciferase activity as compared to Ad5. The

chimpanzee DCs were infected with the GFP viruses and were analysed using a flow cytometer. These results, also shown in figure 29, demonstrate that Ad5 (3%) transduces chimpanzee DCs very poorly as compared to Ad5.Fib35 (66.5%).

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#### Example 11

*Construction of a plasmid-based vector system to generate Ad11-based recombinant viruses*

The results of the neutralization experiments described in Example 5 show that Ad11, like Ad35, was also not neutralized in the vast majority of human serum samples. Therefore, recombinant adenoviruses based on Ad11 are preferred above the commonly used Ad2 and Ad5-based vectors as vectors for gene therapy treatment and vaccination. Both Ad35 and Ad11 are B-group viruses and are classified as viruses belonging to DNA homology cluster 2 (Wadell, 1984). Therefore, the genomes of Ad35 and Ad11 are very similar. To generate a plasmid based system for the production of Ad11-based recombinant viruses the adapter plasmid pAdApt35IP1 generated in Example 7 is modified as follows. Construct pAdApt35IP1 is digested with AvrII and then partially with PacI. The digestion mixture is separated on gel and the 4.4 kb fragment containing the expression cassette and the vector backbone is isolated using the gene clean kit (BIO 101, Inc.). Then a PCR amplification is performed on wtAd11 DNA using the primers 35F1 and 35R2 (see Example 7) using Pwo DNA polymerase according to the manufacturers instructions. The obtained PCR fragment of 0.5 kb is purified using the PCR purification kit (LTI) and ligated to the above prepared fragment of pAdApt35IP1. This gives construct pAdApt11-35IP1 in which the 5' adenovirus fragment is exchanged for the corresponding sequence of Ad11. Next, pAdApt11-35IP1 is digested with BglII and partially with PacI. The obtained fragments are separated on gel and the 3.6 kb fragment containing the vector sequences, the 5' adenovirus fragment and the expression cassette is purified

from gel as above. Next, a PCR fragment is generated using primers 35F3 and 35R4 (see Example 7) on wtAd11 DNA. Amplification is done as above and the obtained 1.3 kb fragment is purified and digested with BglII and PacI. The isolated fragments are then ligated to give construct pAdApt11IP1. This adapter plasmid now contains Ad11 sequences in stead of Ad35 sequences. Correct amplification of PCR amplified Ad11 sequences, is verified by comparison of the sequence in this clone with the corresponding sequence of Ad11 DNA. The latter is obtained by direct sequencing on Ad11 DNA using the indicated PCR primers. The large cosmid clone containing the Ad11 backbone is generated as follows. First, a PCR fragment is amplified on Ad11 DNA using the primers 35F5 and 35R6 with Pwo DNA polymerase as described in Example 7 for Ad35 DNA. The PCR fragment is then purified using the PCR purification kit (LTI) and digested with NotI and NdeI. The resulting 3.1 kb fragment is isolated from gel using the geneclean kit (Bio 101, Inc.). A second PCR fragment is then generated on Ad11 DNA using the primers 35F7 and 35R8 (see Example 7) with Pwo DNA polymerase according to the manufacturers instructions and purified using the PCR purification kit (LTI). This amplified fragment is also digested with NdeI and NotI and the resulting 1.6 kb fragment is purified from gel as above. The two digested PCR fragments are then ligated together with cosmid vector pWE15, previously digested with NotI and dephosphorylated using Tsap enzyme (LTI) according to manufacturers instructions. One clone is selected that has one copy of both fragments inserted. Correct clones are selected by analytical NotI digestion that gives a fragment of 4.7 kb. Confirmation is obtained by a PCR reaction using primers 35F5 and 35R8 that gives a fragment of the same size. The correct clone is then linearized with NdeI and isolated from gel. Next, wtAd11 DNA is digested with NdeI and the large 27 kb fragment is isolated from Low melting point agarose gel using agarase enzyme (Roche) according to

the manufacturers instructions. Both fragments are then ligated and packaged using  $\lambda$  phage packaging extracts (Stratagene) according to the manufacturers protocol. After infection into STBL-2 cells (LTI) colonies are grown on plates and analysed for the presence of the complete insert. The functionality of selected clones is then tested by cotransfection on PER.C6. Hereto, the DNA is digested with NotI and 6  $\mu$ gr is cotransfected with 2  $\mu$ gr of a PCR fragment generated on Ad11 DNA with primers 35F1 and 35R4 (see example 7). Correct clones give CPE within one week following transfection. The correct clone is named pWE.Ad11.pIX-rITR.

Using the above described procedure, a plasmid-based system consisting of an adapter plasmid suitable for insertion of foreign genes and a large helper fragment containing the viral backbone is generated. Recombinant Ad11-based viruses are made using the methods described inhere for Ad35-based recombinant viruses.

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### Example 12

#### *Neutralization of adenoviruses in samples derived from patients*

In the neutralization experiments described in Examples 1 and 5, all samples were derived from healthy volunteers. Since one of the applications of non-neutralized vectors is in the field of gene therapy, it is interesting to investigate whether Ad35 is also neutralized with a low frequency and with low titers in groups of patients that are candidates for treatment with gene therapy.

- Cardio-vascular disease patients

26 paired serum and pericardial fluid (PF) samples were obtained from patients with heart faillure. These were

tested against Ad5 and Ad35 using the neutralization assay described in Example 1. The results confirmed the previous data with samples from healthy volunteers. 70% of the serum samples contained NA to Ad5 and 4% to Ad35. In the  
5 pericardial fluid samples the titers were lower resulting in a total of 40% with NA to Ad5 and none to Ad35. There was a good correlation between NA in PF and serum i.e. there were no positive PF samples without NA in the paired serum sample. These results show that non-neutralized vectors  
10 based on Ad35 are preferred over Ad5 vectors for treatment of cardio-vascular diseases. As is true for all forms of non-neutralized vectors in this application, the vector may be based on the genome of the non-neutralized serotype or may be based on Ad5 (or another serotype) though displaying  
15 at least the major capsid proteins (hexon, penton and optionally fiber) of the non-neutralized serotype.

- Rheumatoid Arthritis patients

The molecular determinant underlying arthritis is not yet  
20 known but both T-cell dysfunction and imbalanced growth factor production in joints is known to cause inflammation and hyperplasia of synovial tissue. The synoviocytes start to proliferate and invade the cartilage and bone which leads to destruction of these tissues. Current treatment starts  
25 (when in an early stage) with administration of anti-inflammatory drugs (anti-TNF, IL1-RA, IL-10) and/or conventional drugs (e.g. MTX, sulfasalazine). In late stage RA synovectomy is performed which is based on surgery, radiation, or chemical intervention. An alternative or  
30 additional option is treatment via gene therapy where an adenoviral vector is delivered directly into the joints of patients and expresses an anti-inflammatory drug or a suicide gene. Previous studies performed in rhesus monkeys suffering from collagen-induced arthritis have shown that  
35 Ad5-based vectors carrying a marker gene can transduce synoviocytes. Whether in the human situation adenoviral

delivery is hampered by the presence of NA is not known. To investigate the presence of NA in synovial fluid (SF) of RA patients, SF samples were obtained from a panel of 53 random selected patients suffering from rheumatoid arthritis (RA).  
5 These were tested against several wt adenoviruses using the neutralization assay as described in Example 1. Results of this screen are presented in Table III. Adenovirus type 5 was found to be neutralized in 72% of the SF samples. Most of these samples contain high titers of NA as also the  
10 highest dilution of the SF sample that was tested (64x) neutralized Ad5 viruses. This means that adenoviral vector delivery to the synoviocytes in the joints of RA patients will be very inefficient. Moreover, since the titers in the SF are so high it is doubtful whether lavage of the joints  
15 prior to vector injection will remove enough of the NA. Of the other serotypes that were tested Ad35 was shown to be neutralized in only 4% of the samples. Therefore, these data confirm the results obtained in serum samples from healthy patients and show that for treatment of rheumatoid arthritis  
20 Ad35-based vectors or chimeric vectors displaying at least some of the capsid proteins from Ad35 are preferred vectors.

### Example 13

#### 25 *Modifications in the backbone of Ad35-based viruses*

##### 1) Generation of pBr/Ad35.Pac-rITR and pBr/Ad35.PRN

Example 4 describes the generation of the Ad35 subclone pBr/Ad35.Eco13.3. This clone contains Ad35 sequences from bp  
30 21943 to the end of the right ITR cloned into the EcoRI and EcoRV sites of pBr322. To extend these sequences to the PacI site located at bp 18137 in Ad35, pBr/Ad35.Eco13.3 (see Example 4) was digested with AatII and SnaBI and the large vector -containing fragment was isolated from gel using the  
35 QIAEX II gel extraction kit (Qiagen). Ad35 wt DNA was digested with PacI and SnaBI and the 4.6 kb fragment was

isolated as above. This fragment was then ligated to a double-stranded (ds) linker containing a PacI and an AatII overhang. This linker was obtained after annealing the following oligonucleotides:

5 A-P1: 5'-CTG GTG GTT AAT-3'

A-P2: 5'-TAA CCA CCA GAC GT-3'

The ligation mix containing the ds linker and the PacI-SnaBI Ad35 fragment was separated from unligated linker on a LMP gel. The 4.6 kb band was cut out the gel, molten at 65 °C, and then ligated to the purified pBr/Ad35.Eco13.3 vector fragment digested with AatII and SnaBI. Ligations were transformed into electrocompetent DH10B cells (Life Technologies Inc.). The resulting clone, pBr/Ad35.Pac-rITR, contained Ad35 sequences from the PacI site at bp 18137 upto the right ITR.

Next, a unique restriction site was introduced at the 3' end or the right ITR to be able to free the ITR from vector sequences. Hereto, a PCR fragment was used that covers Ad35 sequences from the NdeI site at bp 33165 to the right ITR having the restriction sites SmaI, NotI and EcoRI attached to the rITR. The PCR fragment was generated using primers 35F7 and 35R8 (described in example 7). After purification, the PCR fragment was cloned into the AT cloning vector (Invitrogen) and sequenced to verify correct amplification. The correct amplified clone was then digested with EcoRI, blunted with Klenow enzym and subsequently digested with NdeI and the PCR fragment was isolated. In parallel, the NdeI in the pBr vector in pBr/Ad35.Pac-rITR was removed as follows: A pBr322 vector from which the NdeI site was removed by digestion with NdeI, Klenow treatment and religation, was digested with AatII and NheI. The vector fragment was isolated in LMP gel and ligated to the 16.7 kb Ad35 AatII-NheI fragment from pBr/Ad35.Pac-rITR that was also isolated in an LMP gel. This generated pBr/Ad35.Pac-rITR.ΔNdeI. Next pBr/Ad35.Pac-rITR.ΔNdeI was digested with NheI, the ends were filled in using Klenow enzym and the DNA



was then digested with NdeI. The large fragment containing the vector and Ad35 sequences was isolated. Ligation of this vector fragment and the PCR fragment resulted in pBr/Ad35.PRn. In this clone specific sequences coding for fiber, E2A, E3, E4 or hexon can be manipulated. In addition, promoter sequences that drive for instance the E4 proteins or the E2 can be mutated or deleted and exchanged for heterologous promoters.

- 2) Generation of Ad35-based viruses with fiber proteins from different serotypes.

Adenoviruses infect human cells with different efficiencies. Infection is accomplished by a two step process involving: 1. the fiber proteins that mediate binding of the virus to specific receptors on the cells, and 2. the penton proteins that mediate internalization by interaction of for example the RGD sequence to integrins present on the cell surface. For subgroup B viruses of which Ad35 is a member, the cellular receptor for the fiber protein is not known. There are striking differences in infection efficiency of human cells of subgroup B viruses compared to subgroup C viruses like Ad5 (see WO 00/03029 and EP 99200624.7). Even within one subgroup infection efficiencies of certain human cells may differ between various serotypes. For example, the fiber of Ad16, when present on an Ad5-based recombinant virus infects primary endothelial cells, smooth muscle cells and synoviocytes of human and rhesus monkey origin better than Ad5 chimeric viruses carrying the fiber of Ad35 or Ad51. Thus, to obtain high infection efficiencies of Ad35-based viruses, it may be necessary to change the fiber protein for a fiber protein of a different serotype. The technology for such fiber chimeras is described for Ad5-based viruses in Example 3, and is below exemplified for Ad35 viruses.

First, most fiber sequences are deleted from the Ad35 backbone in construct pBr/Ad35.PRn as follows:

The left flanking sequences and part of the fiber protein in Ad35 ranging from bp 30225 upstream of a unique MluI site up to bp 30872 (numbers according to wt Ad35 sequence as disclosed in Figure 6) in the tail of fiber are amplified

5 using primers

DF35-1 : 5'-CAC TCA CCA CCT CCA ATT CC-3'

and

DF35-2: 5'-CGG GAT CCC GTA CGG GTA GAC AGG GTT GAA GG-3'

10 This PCR amplification introduces an unique BsiWI site in the tail of the fiber gene.

The right flanking sequences ranging from the end of the fiber protein at bp 31798 to bp 33199 (numbering according to wtAd35 sequence, Figure 6) , 3' from the unique NdeI site is amplified using primers

15 DF35-3: 5'-CGG GAT CCG CTA GCT GAA ATA AAG TTT AAG TGT TTT TAT TTA AAA TCA C-3'

and

DF35-4: 5'-CCA GTT GCA TTG CTT GGT TGG-3'.

20 This PCR introduces a unique NheI site in the place of the fiber sequences. PCR amplification is done with Pwo DNA polymerase (Roche) according to the manufacturers instructions. After amplification the PCR products are purified using a PCR purification kit and the fragments are digested with BamHI and ligated together. The 2 kb ligated  
25 fragments are purified from gel and cloned in the PCR Script Amp vector (Stratagene). Correct amplification is checked by sequencing. The PCR fragment is then excised as a MluI/NdeI fragment and cloned in pBr/Ad35.PRn digested with the same enzymes. This generates pBr/Ad35.PRAfib, a shuttle vector  
30 suitable to introduce fiber sequences of alternative serotypes. This strategy is analogous to the fiber modification strategy for Ad5-based viruses as disclosed in WO00/03029. Primers that are listed in Table I of that application were used to amplify fiber sequences of various  
35 subgroups of adenovirus. For amplification of fibers that are cloned in the pBr/Ad35.PRAfib the same (degenerate)

primer sequences can be used, however, the NdeI site in the forward primers (tail oligonucleotides A to E) should be changed to a BsiWI site and the NsiI site in the reverse oligo (knob oligonucleotide 1 to 8) should be changed in a NheI site. Thus fiber 16 sequences are amplified using the following degenerate primers:

5'- CCK GTS TAC CCG TAC GAA GAT GAA AGC-3' and 5'-CCG GCT AGC TCA GTC ATC TTC TCT GAT ATA-3'. Amplified sequences are then digested with BsiWI and NheI and cloned into pBr/Ad35.PRAfib digested with the same enzymes to generate pBr/Ad35.PRfib16. The latter construct is then digested with PacI and SwaI and the insert is isolated from gel. The PacI/SwaI Ad35 fragment with modified fiber is then cloned into the corresponding sites of pWE/Ad35.pIX-rITR to give pWE/Ad35.pIX-rITR.fib16. This cosmid backbone can then be used with an Ad35-based adapter plasmid to generate Ad35 recombinant viruses that display the fiber or Ad16. Other fiber sequences can be amplified with (degenerate) primers as mentioned above. If one of the fibers sequences turns out to have an internal BsiWI or NheI site, the PCR fragment has to be digested partially with that enzyme.

### 3) Generation of Ad35-based viruses with inducible, E1 independent, E4 expression.

The adenovirus E4 promoter is activated by expression of E1 proteins. It is not known whether the Ad5 E1 proteins are capable of mediating activation of the Ad35 E4 promoter. Therefore, to enable production of Ad35 recombinant viruses on PER.C6 cells, it may be advantageous to make E4 expression independent of E1. This can be achieved by replacing the Ad35-E4 promoter by heterologous promoter sequences like, but not limited to, the 7xTetO promoter. Recombinant E1-deleted Ad5-based vectors are shown to have residual expression of viral genes from the vector backbone in target cells, despite the absence of E1 expression. Viral gene expression increases the toxicity and may trigger a

host immune response to the infected cell. For most applications of adenoviral vectors in the field of gene therapy and vaccination it is desired to reduce or diminish the expression of viral genes from the backbone. One way to achieve this is to delete all, or as much as possible, sequences from the viral backbone. By deleting E2A, E2B or E4 genes and/or the late gene functions, one has to complement for these functions during production. This complementation can either be by means of a helper virus or through stable addition of these functions, with or without inducible transcription regulation, to the producer cell. Methods to achieve this have been described for Ad5 and are known in the art. One specific method is replacement of the E4 promoter by promoter sequences that are not active in the target cells. E4 proteins play a role in for example replication of adenoviruses through activation of the E2 promoter and in late gene expression through regulation of splicing and nuclear export of late gene transcripts. In addition, at least some of the E4 proteins are toxic to cells. Therefore, reduction or elimination of E4 expression in target cells will further improve Ad35-based vectors. One way to achieve this is to replace the E4 promoter by an heterologous promoter that is inactive in the target cells. An example of a heterologous promoter/activator system that is inactive in target cells is the tetracyclin inducible TetO system (Gossen and Bujard, 1992). Other prokaryotic or synthetic promoter/activator systems may be used. In this example, the E4 promoter in the backbone of the viral vector is replaced by a DNA fragment containing 7 repeats of the tetracyclin responsive element from the tet operon (7xTetO). A strong transactivator for this promoter is a fusion protein containing the DNA binding domain of the tet repressor and the activation domain of VP16 (Tet transactivator protein, Tta). Strong E4 expression, independent of E1 expression, can be accomplished in PER.C6 cells expressing Tta. Tta expressing PER.C6 cells have been

generated and described (see Example 15). Ad5 derived E1-deleted viruses with E4 under control of 7xTetO can be generated and propagated on these cells. Following infection in cells of human or animal origin (that do not express the Tta transactivator), E4 expression was found to be greatly diminished compared to E1 deleted viruses with the normal E4 promoter.

Below the construction of pWE/Ad35.pIX-rITR.TetO-E4, a cosmid helper vector to produce viruses with the E4 promoter replacement, is described.

First, a fragment was generated by PCR amplification on pBr/Ad35.PRn DNA using the following primers:

355ITR: 5'- GAT CCG GAG CTC ACA ACG TCA TTT TCC CAC G-3'  
and

353ITR: 5'-CGG AAT TCG CGG CCG CAT TTA AAT C-3'

This fragment contains sequences between bp 34656 (numbering according to wtAd35) and the NotI site 3' of the right ITR in pBr/Ad35.PRn and introduces an SstI site 5' of the right ITR sequence.

A second PCR fragment was generated on pBr/Ad35.PRn DNA using primers:

35DE4: 5'-CCC AAG CTT GCT TGT GTA TAT ATA TTG TGG-3' and  
35F7: See example 7.

This PCR amplifies Ad35 sequences between bp 33098 and 34500 (numbering according to wtAd35) and introduces a HindIII site upstream of the E4 Tata-box. With these two PCR reactions the right- and left -flanking sequences of the E4 promoter are amplified. For amplification, Pwo DNA polymerase was used according to manufacturers instructions

A third fragment containing the 7xTetO promoter was isolated from construct pAAO-E-TATA-7xTetO by digestion with SstI and HindIII. The generation of pAAO-E-TATA-7xTetO is described below. The first PCR fragment (355/353) was then digested with SstI and NotI and ligated to the 7xTetO fragment. The ligation mixture was then digested with HindIII and NotI and the 0.5 kb fragment is isolated from gel. The second PCR

- fragment (35DE4/35F7) was digested with NdeI and HindIII and gel purified. These two fragments are then ligated into pBr/Ad35.PRn digested with NdeI and NotI to give pBr/Ad35.PR.TetOE4. The modification of the E4 promoter is
- 5 then transferred to the Ad35 helper cosmid clone by exchanging the PacI/SwaI fragment of the latter with the one from pBr/Ad35.PR.TetOE4 to give pWE/Ad35.pIX-rITR.TetOE4. pAAO-E-TATA.7xTetO was generated as follows. Two oligonucleotides were synthesized:
- 10 TATApplus: 5'-AGC TTT CTT ATA AAT TTT CAG TGT TAG ACT AGT AAA TTG CTT AAG-3' and  
TATAmin: 5'-AGC TCT TAA GCA ATT TAC TAG TCT AAC ACT GAA AAT TTA TAA GAA-3'
- (The underlined sequences form a modified TATA box).
- 15 The oligonucleotides were annealed to yield a double stranded DNA fragment with 5' overhangs that are compatible with HindIII digested DNA. The product of the annealing reaction was ligated into HindIII digested pGL3-Enhancer Vector (Promega) to yield pAAO-E-TATA. The clone that had
- 20 the HindIII site at the 5' end of the insert restored was selected for further cloning.
- Next, the heptamerized tet-operator sequence was amplified from the plasmid pUHC-13-3 (Gossen and Bujard, 1992) in a PCR reaction using the Expand PCR system (Roche) according
- 25 to the manufacturers protocol. The following primers were used:
- tet3: 5'- CCG GAG CTC CAT GGC CTA ACT CGA GTT TAC CAC TCC C-3'  
tet5: 5'-CCC AAG CTT AGC TCG ACT TTC ACT TTT CTC-3'
- 30 The amplified fragment was digested with SstI and HindIII (these sites are present in tet3 and tet5 respectively) and cloned into SstI/HindIII digested pAAO-E-TATA giving rise to pAAO-E-TATA-7xtetO
- To test the functionality of the generated pWE/Ad35.pIX-rITR.TetOE4 cosmid clone, the DNA was digested with NotI.
- 35 The left end of wtAd35 DNA was then amplified using primers

35F1 and 35R4 (see example 7). Following amplification, the PCR mixture was purified and digested with SalI to remove intact viral DNA. Then 4gr of both the digested pWE/Ad35.pIX-rITR.TetOE4 and the PCR fragment was  
5 cotransfected into PER.C6-tTA cells that were seeded in T25 flasks the day before. Transfected cells were transferred to T80 flasks after two days and another two days later CPE was obtained, showing that the cosmid backbone is functional.

10

#### Example 14

Generation of cell lines capable of complementing E1-deleted Ad35 viruses

15

#### *Generation of pIG135 and pIG270*

Construct pIG.E1A.E1B contains E1 region sequences of Ad5 corresponding to nucleotides 459 to 3510 of the wt Ad5 sequence (Genbank accession number M72360) operatively linked to the human phosphoglycerate kinase promoter (PGK)  
20 and the Hepatitis B Virus polyA sequences. The generation of this construct is described in WO97/00326. The E1 sequences of Ad5 were replaced by corresponding sequences of Ad35 as follows. pRSV.Ad35-E1 (described in example 8) was digested with EcoRI and Sse8387I and the 3 kb fragment corresponding  
25 to the Ad35 E1 sequences was isolated from gel. Construct pIG.E1A.E1B was digested with Sse8387I completely and partially with EcoRI. The 4.2 kb fragment corresponding to vector sequences without the Ad5 E1 region but retaining the PGK promoter were separated from other fragments on LMP  
30 agarose gel and the correct band was excised from gel. Both obtained fragments were ligated resulting in pIG.Ad35-E1. This vector was further modified to remove the LacZ sequences present in the pUC119 vector backbone. Hereto, the vector was digested with BsaAI and BstXI and the large  
35 fragment was isolated from gel. A double stranded oligo was prepared by annealing the following two oligos:

BB1: 5'-GTG CCT AGG CCA CGG GG-3' and

BB2: 5'-GTG GCC TAG GCA C-3'

Ligation of the oligo and the vector fragment resulted in construct pIG135. Correct insertion of the oligo restores  
5 the BsaAI and BstXI sites and introduces a unique AvrII site. Next, we introduced a unique site at the 3' end of the Ad35-E1 expresion cassette in pIG135. Hereto, the construct was digested with SapI and the 3' protruding ends were made blunt by treatment with T4 DNA polymerase. The thus treated  
10 linear plasmid was further digested with BsrGI and the large vector containing fragment was isolated from gel. To restore the 3' end of the HBVpolyA sequence and to introduce a unique site, a PCR fragment was generated using the following primers:

15 270F: 5'- CAC CTC TGC CTA ATC ATC TC -3' and

270R: 5'- GCT CTA GAA ATT CCA CTG CCT TCC ACC -3'.

The PCR was performed on pIG.Ad35.E1 DNA using Pwo polymerase (Roche) according to the manufacturers instructions. The obtained PCR product was digested with  
20 BsrGI and dephosphorylated using Tsap enzym (LTI), the latter to prevent insert dimerization on the BsrGI site. The PCR fragment and the vector fragment were ligated to yield construct pIG270.

25 *Ad35 E1 sequences are capable of transforming rat primary cells*

New born WAG/RIJ rats were sacrificed at 1 week of gestation and kidneys were isolated. After carefull removal of the capsule, kidneys were disintegrated into a single cell  
30 suspension by multiple rounds of incubation in trypsin/EDTA (LTI) at 37 °C and collection of floating cells in cold PBS containing 1% FBS. When most of the kidney was trypsinized all cells were resuspended in DMEM supplemented with 10% FBS and filtered through a sterile cheese cloth. Baby Rat Kidney  
35 (BRK) cells obtained from one kidney were plated in 5 dishes (Greiner, 6 cm). When a confluency of 70-80% was reached,



the cells were transfected with 1 or 5  $\mu$ gr DNA/dish using the  $\text{CaPO}_4$  precipitation kit (LTI) according to the manufacturers instructions. The following constructs were used in separate transfections: pIG.E1A.E1B (expressing the Ad5-E1 region), pRSV.Ad35-E1, pIG.Ad35-E1 and pIG270 (the latter expressing the Ad35-E1). Cells were incubated at 37 °C, 5%  $\text{CO}_2$  until foci of transformed cells appeared. Table IV shows the number of foci that resulted from several transfection experiments using circular or linear DNA. As expected, the Ad5-E1 region efficiently transformed BRK cells. Foci also appeared in the Ad35-E1 transfected cell layer although with lower efficiency. The Ad35 transformed foci appeared at a later time point: ~2 weeks post transfection compared with 7-10 days for Ad5-E1. These experiments clearly show that the E1 genes of the B group virus Ad35 are capable of transforming primary rodent cells. This proves the functionality of the Ad35-E1 expression constructs and confirms earlier findings of the transforming capacity of the B-group viruses Ad3 and Ad7 (Dijkema, 1979). To test whether the cells in the foci were really transformed a few foci were picked and expanded. From the 7 picked foci at least 5 turned out to grow as established cell lines.

25 *Generation of new packaging cells derived from primary human amniocytes*

Amniotic fluid obtained after amnioscentesis was centrifugated and cells were resuspended in AmnioMax medium (LTI) and cultured in tissue culture flasks at 37 °C and 10 %  $\text{CO}_2$ . When cells were growing nicely (approximately one cell division/24 hrs.), the medium was replaced with a 1:1 mixture of AmnioMax complete medium and DMEM low glucose medium (LTI) supplemented with Glutamax I (end concentration 4mM, LTI) and glucose (end concentration 4.5 gr/L, LTI) and 10% FBS (LTI). For transfection ~  $5 \times 10^5$  cells were plated in 10 cm tissue culture dishes. The day after, cells were

transfected with 20  $\mu$ gr of circular pIG270/dish using the CaPO<sub>4</sub> transfection kit (LTI) according to manufacturers instructions and cells were incubated overnight with the DNA precipitate. The following day, cells were washed 4 times  
5 with PBS to remove the precipitate and further incubated for over three weeks until foci of transformed cells appeared. Once a week the medium was replaced by fresh medium. Other transfection agents like, but not limited to, LipofectAmine (LTI) or PEI (Polyethylenimine, high molecular weight,  
10 water-free, Aldrich) were used. Of these three agents PEI reached the best transfection efficiency on primary human amniocytes: ~1% blue cells 48 hrs. following transfection of pAdApt35.LacZ.

Foci are isolated as follows. The medium is removed and  
15 replaced by PBS after which foci are isolated by gently scraping the cells using a 50-200  $\mu$ l Gilson pipette with a disposable filter tip. Cells contained in 10  $\mu$ l PBS were brought in a 96 well plate containing 15  $\mu$ l trypsin/EDTA (LTI) and a single cell suspension was obtained by pipetting  
20 up and down and a short incubation at room temperature. After addition of 200  $\mu$ l of the above described 1:1 mixture of AmnioMax complete medium and DMEM with supplements and 10% FBS, cells were further incubated. Clones that continued to grow were expanded and analysed their ability to  
25 complement growth of E1-deleted adenoviral vectors of different sub-groups, specifically ones derived from B-group viruses specifically from Ad35 or Ad11.

30 *Generation of new packaging cell lines from human embryonic retinoblasts*

Human retina cells are isolated from the eyes of aborted foetuses and cultured in DMEM medium (LTI) supplemented with 10% FBS (LTI). The day before transfection,  $\sim 5 \times 10^5$  cells are plated in 6 cm dishes and cultured overnight at 37 °C and  
35 10% CO<sub>2</sub>. Transfection is done using the CaPO<sub>4</sub> precipitation kit (LTI) according to the manufacturers instructions. Each

dish is transfected with 8-10 µgr pIG270 DNA, either as a circular plasmid or as a purified fragment. To obtain the purified fragment, pIG270 was digested with AvrII and XbaI and the 4 kb fragment corresponding to the Ad35 E1 expression cassette was isolated from gel by agarase treatment (Roche). The following day, the precipitate is washed away carefully by four washes with sterile PBS. Then fresh medium is added and transfected cells are further cultured until foci of transformed cells appear. When large enough (>100 cells) foci are picked and brought into 96-wells as described above. Clones of transformed human embryonic retinoblasts that continue to grow, are expanded and tested for their ability to complement growth of E1-deleted adenoviral vectors of different sub-groups specifically ones derived from B-group viruses specifically from Ad35 or Ad11.

*New packaging cell lines derived from PER.C6*

As described in example 8, it is possible to generate and grow Ad35 E1-deleted viruses on PER.C6 cells with cotransfection of an Ad35-E1 expression construct, e.g. pRSV.Ad35.E1. However, large scale production of recombinant adenoviruses using this method is cumbersome because for each amplification step a transfection of the Ad35-E1 construct is needed. In addition, this method increases the risk of non-homologous recombination between the plasmid and the virus genome with high chances of generation of recombinant viruses that incorporate E1 sequences resulting in replication competent viruses. To avoid this, the expression of Ad35-E1 proteins in PER.C6 has to be mediated by integrated copies of the expression plasmid in the genome. Since PER.C6 cells are already transformed and express Ad5-E1 proteins, addition of extra Ad35-E1 expression may be toxic for the cells, however, it is not impossible to stably transfect transformed cells with E1

proteins since Ad5-E1 expressing A549 cells have been generated..

In an attempt to generate recombinant adenoviruses derived from subgroup B virus Ad7, Abrahamsen et al. (1997) were not  
5 able to generate E1-deleted viruses on 293 cells without contamination of wt Ad7. Viruses that were picked after plaque purification on 293-ORF6 cells (Brough et al., 1996) were shown to have incorporated Ad7 E1B sequences by non-homologous recombination. Thus, efficient propagation of Ad7  
10 recombinant viruses proved possible only in the presence of Ad7-E1B expression and Ad5-E4-ORF6 expression. The E1B proteins are known to interact with cellular as well as viral proteins (Bridge et al., 1993; White, 1995). Possibly, the complex formed between the E1B 55K protein and E4-ORF6 which  
15 is necessary to increase mRNA export of viral proteins and to inhibit export of most cellular mRNAs, is critical and in some way serotype specific. The above experiments suggest that the E1A proteins of Ad5 are capable of complementing an Ad7-E1A deletion and that Ad7-E1B expression in adenovirus  
20 packaging cells on itself is not enough to generate a stable complementing cell line. To test whether one or both of the Ad35-E1B proteins is/are the limiting factor in efficient Ad35 vector propagation on PER.C6 cells, we have generated an Ad35 adapter plasmid that does contain the E1B promoter  
25 and E1B sequences but lacks the promoter and the coding region for E1A. Hereto, the left end of wtAd35 DNA was amplified using the primers 35F1 and 35R4 (both described in Example 7) with Pwo DNA polymerase (Roche) according to the manufacturers' instructions. The 4.6 kb PCR product was  
30 purified using the PCR purification kit (LTI) and digested with SnaBI and ApaI enzymes. The resulting 4.2 kb fragment was then purified from gel using the QIAExII kit (Qiagen). Next, pAdApt35IP1 (Example 7) was digested with SnaBI and ApaI and the 2.6 kb vector containing fragment was isolated  
35 from gel using the GeneClean kit (BIO 101, Inc). Both

isolated fragments were ligated to give pBr/Ad35.leftITR-pIX. Correct amplification during PCR was verified by a functionality test as follows: The DNA was digested with BstBI to liberate the Ad35 insert from vector sequences and 4 µgr of this DNA was cotransfected with 4 µgr of NotI digested pWE/Ad35.pIX-rITR (Example 7) into PER.C6 cells. The transfected cells were passaged to T80 flasks at day 2 and again two days later CPE had formed showing that the new pBr/Ad35.leftITR-pIX construct contains functional E1 sequences. The pBr/Ad35.leftITR-pIX construct was then further modified as follows. The DNA was digested with SnaBI and HindIII and the 5' HindII overhang was filled in using Klenow enzyme. Religation of the digested DNA and transformation into competent cells (LTI) gave construct pBr/Ad35leftITR-pIXΔE1A. This latter construct contains the left end 4.6 kb of Ad35 except for E1A sequences between bp 450 and 1341 (numbering according to wtAd35, Figure 6) and thus lacks the E1A promoter and most of the E1A coding sequences. pBr/Ad35.leftITR-pIXΔE1A was then digested with BstBI and 2 µgr of this construct was cotransfected with 6 µgr of NotI digested pWE/Ad35.pIX-rITR (Example 7) into PER.C6 cells. One week following transfection full CPE had formed in the transfected flasks.

This experiment shows that the Ad35-E1A proteins are functionally complemented by Ad5-e1A expression in PER.C6 cells and that at least one of the Ad35-E1B proteins cannot be complemented by Ad5-E1 expression in PER.C6. It further shows that it is possible to make a complementing cell line for Ad35 E1-deleted viruses by expressing Ad35-E1B proteins in PER.C6. Stable expression of Ad35-E1B sequences from integrated copies in the genome of PER.C6 cells may be driven by the E1B promoter and terminated by a heterologous poly-adenylation signal like, but not limited to, the HBVpA. The heterologous pA signal is necessary to avoid overlap between the E1B insert and the recombinant vector, since the natural E1B termination is located in the pIX transcription

unit which has to be present on the adenoviral vector.  
Alternatively, the E1B sequences may be driven by a  
heterologous promoter like, but not limited to the human PGK  
promoter or by an inducible promoter like, but not limited to  
5 the 7xtetO promoter (Gossen and Bujard, 1992). Also in these  
cases the transcription termination is mediated by a  
heterologous pA sequence, e.g. the HBV pA. The Ad35-E1B  
sequences at least comprise one of the coding regions of the  
E1B 21K and the E1B 55K proteins located between nucleotides  
10 1611 and 3400 of the wt Ad35 sequence. The insert may also  
include (part of the) Ad35-E1B sequences between nucleotides  
1550 and 1611 of the wt Ad35 sequence.

#### Example 15

- 15 Generation of producer cell lines for the production of  
recombinant adenoviral vectors deleted in early region 1 and  
early region 2A

##### *Generation of PER.C6-tTA cells*

- 20 Here is described the generation of cell lines for the  
production of recombinant adenoviral vectors that are  
deleted in early region 1 (E1) and early region 2A (E2A).  
The producer cell lines complement for the E1 and E2A  
deletion from recombinant adenoviral vectors *in trans* by  
25 constitutive expression of both E1 and E2A genes. The pre-  
established Ad5-E1 transformed human embryo retinoblast cell  
line PER.C6 (WO 97/00326) was further equipped with E2A  
expression cassettes.

- The adenoviral E2A gene encodes a 72 kDa DNA Binding  
30 Protein with has a high affinity for single stranded DNA.  
Because of its function, constitutive expression of DBP is  
toxic for cells. The ts125E2A mutant encodes a DBP which has  
a Pro→Ser substitution of amino acid 413. Due to this  
mutation, the ts125E2A encoded DBP is fully active at the  
35 permissive temperature of 32°C, but does not bind to ssDNA  
at the non-permissive temperature of 39°C. This allows the

generation of cell lines that constitutively express E2A, which is not functional and is not toxic at the non-permissive temperature of 39°C. Temperature sensitive E2A gradually becomes functional upon temperature decrease and becomes fully functional at a temperature of 32°C, the permissive temperature.

A. Generation of plasmids expressing the wild type E2A- or temperature sensitive ts125E2A gene.

10        pcDNA3wtE2A: The complete wild-type early region 2A (E2A) coding region was amplified from the plasmid pBR/Ad.Bam-rITR (ECACC deposit P97082122) with the primers DBPpcr1 and DBPpcr2 using the Expand™ Long Template PCR system according to the standard protocol of the supplier  
15 (Boehringer Mannheim). The PCR was performed on a Biometra Trio Thermoblock, using the following amplification program: 94°C for 2 minutes, 1 cycle; 94°C for 10 seconds + 51°C for 30 seconds + 68°C for 2 minutes, 1 cycle; 94°C for 10 seconds + 58°C for 30 seconds + 68°C for 2 minutes, 10  
20 cycles; 94°C for 10 seconds + 58°C for 30 seconds + 68°C for 2 minutes with 10 seconds extension per cycle, 20 cycles; 68°C for 5 minutes, 1 cycle. The primer DBPpcr1: CGG GAT CCG CCA CCA TGG CCA GTC GGG AAG AGG AG (5' to 3') contains a unique *Bam*HI restriction site (underlined) 5' of the Kozak  
25 sequence (*italic*) and start codon of the E2A coding sequence. The primer DBPpcr2: CGG AAT TCT TAA AAA TCA AAG GGG TTC TGC CGC (5' to 3') contains a unique *Eco*RI restriction site (underlined) 3' of the stop codon of the E2A coding sequence. The bold characters refer to sequences  
30 derived from the E2A coding region. The PCR fragment was digested with *Bam*HI/*Eco*RI and cloned into *Bam*HI/*Eco*RI digested pcDNA3 (Invitrogen), giving rise to pcDNA3wtE2A.

pcDNA3tsE2A: The complete ts125E2A-coding region was amplified from DNA isolated from the temperature sensitive  
35 adenovirus mutant H5ts125. The PCR amplification procedure

was identical to that for the amplification of wtE2A. The PCR fragment was digested with *Bam*HI/*Eco*RI and cloned into *Bam*HI/*Eco*RI digested pcDNA3 (Invitrogen), giving rise to pcDNA3tsE2A. The integrity of the coding sequence of wtE2A and tsE2A was confirmed by sequencing.

B. Growth characteristics of producer cells for the production of recombinant adenoviral vectors cultured at 32-, 37- and 39°C.

PER.C6 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL) supplemented with 10% Fetal Bovine Serum (FBS, Gibco BRL) and 10mM MgCl<sub>2</sub> in a 10% CO<sub>2</sub> atmosphere at either 32°C, 37°C or 39°C. At day 0, a total of 1 x 10<sup>6</sup> PER.C6 cells were seeded per 25cm<sup>2</sup> tissue culture flask (Nunc) and the cells were cultured at either 32°C, 37°C or 39°C. At day 1-8, cells were counted. Figure 30 shows that the growth rate and the final cell density of the PER.C6 culture at 39°C are comparable to that at 37°C. The growth rate and final density of the PER.C6 culture at 32°C were slightly reduced as compared to that at 37°C or 39°C. No significant cell death was observed at any of the incubation temperatures. Thus PER.C6 performs very well both at 32°C and 39°C, the permissive and non-permissive temperature for ts125E2A, respectively.

C. Transfection of PER.C6 with E2A expression vectors; colony formation and generation of cell lines

One day prior to transfection, 2 x 10<sup>6</sup> PER.C6 cells were seeded per 6 cm tissue culture dish (Greiner) in DMEM, supplemented with 10% FBS and 10mM MgCl<sub>2</sub> and incubated at 37°C in a 10% CO<sub>2</sub> atmosphere. The next day, the cells were transfected with 3, 5 or 8µg of either pcDNA3, pcDNA3wtE2A or pcDNA3tsE2A plasmid DNA per dish, using the LipofectAMINE PLUS™ Reagent Kit according to the standard protocol of the supplier (Gibco BRL), except that the cells were transfected at 39°C in a 10% CO<sub>2</sub> atmosphere. After the transfection, the



cells were constantly kept at 39°C, the non-permissive temperature for ts125E2A. Three days later, the cells were put in DMEM supplemented with 10% FBS, 10mM MgCl<sub>2</sub> and 0.25mg/ml G418 (Gibco BRL), and the first G418 resistant colonies appeared at 10 days post transfection. As shown in table 1, there was a dramatic difference between the total number of colonies obtained after transfection of pcDNA3 (~200 colonies) or pcDNA3tsE2A (~100 colonies) and pcDNA3wtE2A (only 4 colonies). These results indicate that the toxicity of constitutively expressed E2A can be overcome by using a temperature sensitive mutant of E2A (ts125E2A) and culturing of the cells at the non-permissive temperature of 39°C.

From each transfection, a number of colonies was picked by scraping the cells from the dish with a pipette. The detached cells were subsequently put into 24 wells tissue culture dishes (Greiner) and cultured further at 39°C in a 10% CO<sub>2</sub> atmosphere in DMEM, supplemented with 10% FBS, 10mM MgCl<sub>2</sub> and 0.25mg/ml G418. As shown in table 1, 100% of the pcDNA3 transfected colonies (4/4) and 82% of the pcDNA3tsE2A transfected colonies (37/45) were established to stable cell lines (the remaining 8 pcDNA3tsE2A transfected colonies grew slowly and were discarded). In contrast, only 1 pcDNA3wtE2A-transfected colony could be established. The other 3 died directly after picking.

Next, the E2A expression levels in the different cell lines were determined by Western blotting. The cell lines were seeded on 6 well tissue culture dishes and sub-confluent cultures were washed twice with PBS (NPBI) and lysed and scraped in RIPA (1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS in PBS, supplemented with 1mM phenylmethylsulfonylfluoride and 0.1 mg/ml trypsin inhibitor). After 15 minutes incubation on ice, the lysates were cleared by centrifugation. Protein concentrations were determined by the Bio-Rad protein assay, according to standard procedures of the supplier (BioRad). Equal amounts

of whole-cell extract were fractionated by SDS-PAGE on 10% gels. Proteins were transferred onto Immobilon-P membranes (Millipore) and incubated with the  $\alpha$ DBP monoclonal antibody B6. The secondary antibody was a horseradish-peroxidase-conjugated goat anti mouse antibody (BioRad). The Western blotting procedure and incubations were performed according to the protocol provided by Millipore. The complexes were visualized with the ECL detection system according to the manufacturer's protocol (Amersham). Figure 31 shows that all of the cell lines derived from the pcDNA3tsE2A transfection expressed the 72-kDa E2A protein (left panel, lanes 4-14; middle panel, lanes 1-13; right panel, lanes 1-12). In contrast, the only cell line derived from the pcDNAwtE2A transfection did not express the E2A protein (left panel, lane 2). No E2A protein was detected in extract from a cell line derived from the pcDNA3 transfection (left panel, lane 1), which served as a negative control. Extract from PER.C6 cells transiently transfected with pcDNA3ts125 (left panel, lane 3) served as a positive control for the Western blot procedure. These data confirmed that constitutive expression of wtE2A is toxic for cells and that using the ts125 mutant of E2A could circumvent this toxicity.

D. Complementation of E2A deletion in adenoviral vectors on PER.C6 cells constitutively expressing full-length ts125E2A. The adenovirus Ad5.dl802 is an Ad 5 derived vector deleted for the major part of the E2A coding region and does not produce functional DBP. Ad5.dl802 was used to test the E2A trans-complementing activity of PER.C6 cells constitutively expressing ts125E2A. Parental PER.C6 cells or PER.C6tsE2A clone 3-9 were cultured in DMEM, supplemented with 10% FBS and 10mM MgCl<sub>2</sub>, at 39°C and 10% CO<sub>2</sub> in 25 cm<sup>2</sup> flasks and either mock infected or infected with Ad5.dl802 at an m.o.i. of 5. Subsequently the infected cells were cultured at 32°C and cells were screened for the appearance of a cytopathic effect (CPE) as determined by changes in cell morphology and

detachment of the cells from the flask. Full CPE appeared in the Ad5.dl802 infected PER.C6tsE2A clone 3-9 within 2 days. No CPE appeared in the Ad5.dl802 infected PER.C6 cells or the mock infected cells. These data showed that PER.C6 cells  
5 constitutively expressing ts125E2A complemented in trans for the E2A deletion in the Ad5.dl802 vector at the permissive temperature of 32°C.

E. Serum-free suspension culture of PER.C6tsE2A cell lines.  
10 Large-scale production of recombinant adenoviral vectors for human gene therapy requires an easy and scalable culturing method for the producer cell line, preferably a suspension culture in medium devoid of any human or animal  
15 constituents. To that end, the cell line PER.C6tsE2A c5-9 (designated c5-9) was cultured at 39°C and 10% CO<sub>2</sub> in a 175 cm<sup>2</sup> tissue culture flask (Nunc) in DMEM, supplemented with 10% FBS and 10mM MgCl<sub>2</sub>. At sub-confluency (70-80%  
20 confluent), the cells were washed with PBS (NPBI) and the medium was replaced by 25 ml serum free suspension medium Ex-cell™ 525 (JRH) supplemented with 1 x L-Glutamine (Gibco BRL), hereafter designated SFM. Two days later, cells were  
detached from the flask by flicking and the cells were centrifuged at 1,000 rpm for 5 minutes. The cell pellet was resuspended in 5 ml SFM and 0.5 ml cell suspension was  
25 transferred to a 80 cm<sup>2</sup> tissue culture flask (Nunc), together with 12 ml fresh SFM. After 2 days, cells were harvested (all cells are in suspension) and counted in a  
Burker cell counter. Next, cells were seeded in a 125 ml tissue culture erlenmeyer (Corning) at a seeding density of  
30 3 x 10<sup>5</sup> cells per ml in a total volume of 20 ml SFM. Cells were further cultured at 125 RPM on an orbital shaker (GFL) at 39°C in a 10% CO<sub>2</sub> atmosphere. Cells were counted at day  
1-6 in a Burker cell counter. In Figure 4, the mean growth curve from 8 cultures is shown. PER.C6tsE2A c5-9 performed  
35 well in serum free suspension culture. The maximum cell

density of approximately  $2 \times 10^6$  cells per ml is reached within 5 days of culture.

F. Growth characteristics of PER.C6 and PER.C6/E2A at 37°C and 39°C.

PER.C6 cells or PER.C6ts125E2A (c8-4) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL) supplemented with 10% Fetal Bovine Serum (FBS, Gibco BRL) and 10mM MgCl<sub>2</sub> in a 10% CO<sub>2</sub> atmosphere at either 37°C (PER.C6) or 39°C (PER.C6ts125E2A c8-4). At day 0, a total of  $1 \times 10^6$  cells were seeded per 25cm<sup>2</sup> tissue culture flask (Nunc) and the cells were cultured at the respective temperatures. At the indicated time points, cells were counted. The growth of PER.C6 cells at 37°C was comparable to the growth of PER.C6ts125E2A c8-4 at 39°C (Figure 33). This shows that constitutive expression of ts125E2A encoded DBP had no adverse effect on the growth of cells at the non-permissive temperature of 39°C.

G. Stability of PER.C6ts125E2A

For several passages, the PER.C6ts125E2A cell line clone 8-4 was cultured at 39°C and 10% CO<sub>2</sub> in a 25 cm<sup>2</sup> tissue culture flask (Nunc) in DMEM, supplemented with 10% FBS and 10 mM MgCl<sub>2</sub> in the absence of selection pressure (G418). At sub-confluency (70-80% confluent), the cells were washed with PBS (NPBI) and lysed and scraped in RIPA (1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS in PBS, supplemented with 1mM phenylmethylsulfonylfluoride and 0.1 mg/ml trypsin inhibitor). After 15 minutes incubation on ice, the lysates were cleared by centrifugation. Protein concentrations were determined by the BioRad protein assay, according to standard procedures of the supplier (BioRad). Equal amounts of whole-cell extract were fractionated by SDS-PAGE in 10% gels. Proteins were transferred onto Immobilon-P membranes (Millipore) and incubated with the  $\alpha$ DBP monoclonal antibody B6. The secondary antibody was a horseradish-peroxidase-

conjugated goat anti mouse antibody (BioRad). The Western blotting procedure and incubations were performed according to the protocol provided by Millipore. The complexes were visualized with the ECL detection system according to the manufacturer's protocol (Amersham). The expression of ts125E2A encoded DBP was stable for at least 16 passages, which is equivalent to approximately 40 cell doublings (Figure 34). No decrease in DBP levels was observed during this culture period, indicating that the expression of ts125E2A was stable, even in the absence of G418 selection pressure.

#### Example 16

##### Generation of tTA expressing packaging cell lines

15

A. Generation of a plasmid from which the tTA gene is expressed.

pcDNA3.1-tTA: The tTA gene, a fusion of the tetR and VP16 genes, was removed from the plasmid pUHD 15-1 (Gossen and Bujard, 1992) by digestion using the restriction enzymes BamHI and EcoRI. First, pUHD15-1 was digested with EcoRI. The linearized plasmid was treated with Klenow enzyme in the presence of dNTPs to fill in the EcoRI sticky ends. Then, the plasmid was digested with BamHI. The resulting fragment, 1025 bp in length, was purified from agarose. Subsequently, the fragment was used in a ligation reaction with BamHI/EcoRV digested pcDNA 3.1 HYGRO (-) (Invitrogen) giving rise to pcDNA3.1-tTA. After transformation into competent *E. Coli* DH5 $\alpha$  (Life Techn.) and analysis of ampiciline resistant colonies, one clone was selected that showed a digestion pattern as expected for pcDNA3.1-tTA.

*B. Transfection of PER.C6 and PER.C6/E2A with the tTA expression vector; colony formation and generation of cell lines*

5           One day prior to transfection,  $2 \times 10^6$  PER.C6 or  
PER.C6/E2A cells were seeded per 60 mm tissue culture dish  
(Greiner) in Dulbecco's modified essential medium (DMEM,  
Gibco BRL) supplemented with 10% FBS (JRH) and 10 mM  $MgCl_2$ ,  
and incubated at 37°C in a 10%  $CO_2$  atmosphere. The next day,  
10 cells were transfected with 4-8  $\mu g$  of pcDNA3.1-tTA plasmid  
DNA using the LipofectAMINE PLUS™ Reagent Kit according to  
the standard protocol of the supplier (Gibco BRL). The cells  
were incubated with the LipofectAMINE PLUS™-DNA mixture for  
four hours at 37°C and 10%  $CO_2$ . Then, 2 ml of DMEM  
15 supplemented with 20% FBS and 10 mM  $MgCl_2$ , was added and  
cells were further incubated at 37°C and 10%  $CO_2$ . The next  
day, cells were washed with PBS and incubated in fresh DMEM  
supplemented with 10% FBS, 10 mM  $MgCl_2$ , at either 37°C  
(PER.C6) or 39°C (Per.C6/E2A) in a 10%  $CO_2$  atmosphere for  
20 three days. Then, the media were exchanged for selection  
media; PER.C6 cells were incubated with DMEM supplemented  
with 10% FBS, 10 mM  $MgCl_2$ , and 50  $\mu g/ml$  hygromycin B (GIBCO)  
while PER.C6/E2A cells were maintained in DMEM supplemented  
with 10% FBS, 10 mM  $MgCl_2$ , and 100  $\mu g/ml$  hygromycin B.  
25 Colonies of cells that resisted the selection appeared  
within three weeks while nonresistant cells died during this  
period.

From each transfection, a number of independent,  
hygromycin resistant cell colonies were picked by scraping  
30 the cells from the dish with a pipette and put into 2.5  $cm^2$   
dishes (Greiner) for further growth in DMEM containing 10%  
FBS, 10 mM  $MgCl_2$ , and supplemented with 50  $\mu g/ml$  (PERC.6  
cells) or 100  $\mu g/ml$  (PERC.6/E2A cells) hygromycin in a 10%  
 $CO_2$  atmosphere and at 37°C or 39°C, respectively.

Next, it was determined whether these hygromycin-resistant cell colonies expressed functional tTA protein. Therefore, cultures of PER.C6/tTA or PER/E2A/tTA cells were transfected with the plasmid pUHC 13-3 that contains the reporter gene luciferase under the control of the 7xtetO promoter (Gossens and Bujard, 1992). To demonstrate that the expression of luciferase was mediated by tTA, one half of the cultures was maintained in medium without doxycycline. The other half was maintained in medium with 8 µg/ml doxycycline (Sigma). The latter drug is an analogue of tetracycline and binds to tTA and inhibits its activity. All PER.C6/tTA and PER/E2A/tTA cell lines yielded high levels of luciferase, indicating that all cell lines expressed the tTA protein (Figure 35). In addition, the expression of luciferase was greatly suppressed when the cells were treated with doxycycline. Collectively, the data showed that the isolated and established hygromycin-resistant PER.C6 and PER/E2A cell clones all expressed functional tTA.

Legend to the figures:

## Figure 1:

- 5 Bar graph showing the percentage of serum samples positive for neutralisation for each human wt adenovirus tested (see example1 for description of the neutralisation assay).

## Figure 2:

- 10 Graph showing absence of correlation between the VP/CCID50 ratio and the percentage of neutralisation.

## Figure 3:

- 15 Schematic representation of a partial restriction map of Ad35 (taken from Kang et al., 1989) and the clones generated to make recombinant Ad35-based viruses.

Figure 4: Bar graph presenting the percentage sera samples that show neutralizing activity to a selection of adenovirus serotypes. Sera were derived from healthy volunteers from Belgium and the UK.

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- Figure 5: Bar graph presenting the percentage sera samples that show neutralizing activity to adenovirus serotypes 5, 11, 26, 34, 35, 48 and 49. Sera were derived from five different locations in Europe and the United States.
- 25

- Figure 6: Sequence of human adenovirus type 35. As explained in the text the nucleotide sequence of the terminal ends of the virus are not definite resolved.
- 30

Figure 7: Map of pAdApt

Figure 8: Map of pIPspAdapt

Figure 9: Map of pIPspAdapt1

Figure 10: Map of pIPspAdapt3

- 35 Figure 11: Map of pAdApt35IP3



- Figure 12: Map of pAdApt35IP1
- Figure 13: Schematic representation of the steps undertaken to construct pWE.Ad35.pIX-rITR
- Figure 14: Map of pWE.Ad35.pIX-rITR
- 5 Figure 15: Map of pRSV.Ad35-E1
- Figure 16: Map of PGKneopA
- Figure 17: Map of pRSVpNeo
- Figure 18: Map of pRSVhbtNeo
- 10 Figure 19: Flow cytometric analyses on Green fluorescent protein (GFP) expression in human TF-1 cells. Non-transduced TF-1 cells were used to set a background level of 1%. GFP expression in cells transduced with Ad5, Ad5.Fib16, Ad5.Fib17, Ad5.Fib40-L, Ad5.Fib35, and Ad5.Fib51 is shown.
- 15 Figure 20: Transduction of primary human fibroblast-like stroma. Cells were analyzed 48 hours after a two hour exposure to the different chimaeric fiber viruses. Shown is percentage of cells found positive for the transgene: green fluorescent protein (GFP) using a flow cytometer. Non-transduced stroma cells were used to set a background at 1%. Results of different experiments (n=3) are shown  $\pm$  standard deviation.
- 20 Figure 21: Transduction of primary human fibroblast-like stroma, CD34<sup>+</sup> cells and CD34<sup>+</sup>Lin<sup>-</sup> cells. Cells were analyzed 5 days after a two hour exposure to the different chimaeric fiber viruses. Shown is percentage of cells found positive for the transgene: green fluorescent protein (GFP) using a flow cytometer. Non-transduced cells were used to set a background at 1%. Also shown is the number of GFP positive events divided by the total number of events analysed (between brackets).
- 25 Figure 22 A) Flow cytometric analysis of GFP positive cells after transduction of CD34<sup>+</sup> cells with Ad5.Fib51. All cells

gated in R2-R7 are positive for CD34 but differ in their expression of early differentiation markers CD33, CD38, and CD71 (Lin). Cells in R2 are negative for CD33, CD38, CD71 whereas cells in R7 are positive for these markers. To demonstrate specificity of Ad5.Fib51 the percentage of GFP positive cells was determined in R2-R7 which proved to decline from 91% (R2) to 15% (R7). B) Identical experiment as shown under A (X-axis is R2-R7) but for the other Ad fiber chimeric viruses showing that Ad5.Fib35, and Ad5.Fib16 behave similar as Ad5.Fib51.

Figure 23: Alignment of the chimeric fiber proteins of Ad5fib16, Ad5fib35 and Ad5fib51 with the Ad5 fiber sequence.

Figure 24: Toxicity of Adenovirus exposure to primitive human Bone marrow cells and Stem cells. Cell cultures were counted just before and 5 days after adenovirus transduction. Shown is the percentage of primitive human bone marrow cells (CD34<sup>+</sup>) and HSCs (CD34<sup>+</sup>Lin<sup>-</sup>) recovered as compared to day 0.

Figure 25: Transduction of immature DCs at a virus dose of 100 or 1000 virus particles per cell. Virus tested is Ad5 and Ad5 based vectors carrying the fiber of serotype 12 (Ad5.Fib12), 16 (Ad5.Fib16), 28 (Ad5.Fib28), 32 (Ad5.Fib32), the long fiber of 40 (Ad5.Fib40-L, 49 (Ad5.Fib49), 51 (Ad5.Fib51). Luciferase transgene expression is expressed as relative light units per microgram of protein.

Figure 26: Flow cytometric analyses of LacZ expression on immature and mature DCs transduced with 10000 virus particles per cell of Ad5 or the fiber chimeric vectors Ad5.Fib16, Ad5.Fib40-L, or Ad5.Fib51. Percentages of cells scored positive are shown in upper left corner of each histogram.

Figure 27: Luciferase transgene expression in human immature DCs measured 48 hours after transduction with 1000 or 5000 virus particles per cell. Virus tested were fiber chimaeric viruses carrying the fiber of subgroup B members (serotypes 11, 16, 35, and 51).

Figure 28: Green fluorescent protein (GFP) expression in immature human DCs 48 hours after transduction with 1000 virus particles per cell of Ad5, Ad5.Fib16, and Ad5.Fib35. Non-transduced cells were used to set a background level of approximately 1% (-).

Figure 29: Transduction of mouse and chimpanzee DCs. Luciferase transgene expression measured in mouse DCs 48 hours after transduction is expressed as relative light units per microgram of protein. Chimpanzee DCs were measured 48 hours after transduction using a flow cytometer. GFP expression demonstrates the poor transduction of Ad (35) in contrast to Ad5.Fib35 (66%).

Figure 30: Temperature dependent growth of PER.C6. PER.C6 cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% Fetal Bovine Serum (FBS, Gibco BRL) and 10mM MgCl<sub>2</sub> in a 10% CO<sub>2</sub> atmosphere at either 32°C, 37°C or 39°C. At day 0, a total of 1 x 10<sup>6</sup> PER.C6 cells were seeded per 25cm<sup>2</sup> tissue culture flask (Nunc) and the cells were cultured at either 32°C, 37°C or 39°C. At day 1-8, cells were counted. The growth rate and the final cell density of the PER.C6 culture at 39°C are comparable to that at 37°C. The growth rate and final density of the PER.C6 culture at 32°C were slightly reduced as compared to that at 37°C or 39°C.

PER.C6 cells were seeded at a density of 1 x 10<sup>6</sup> cells per 25 cm<sup>2</sup> tissue culture flask and cultured at either 32-, 37- or 39°C. At the indicated time points, cells were counted in

a Burkner cell counter. PER.C6 grows well at both 32-, 37- and 39<sup>0</sup>C.

Figure 31: DBP levels in PER.C6 cells transfected with pcDNA3, pcDNA3wtE2A or pcDNA3ts125E2A.

Equal amounts of whole-cell extract were fractionated by SDS-PAGE on 10% gels. Proteins were transferred onto Immobilon-P membranes and DBP protein was visualized using the  $\alpha$ DBP monoclonal B6 in an ECL detection system. All of the cell lines derived from the pcDNA3ts125E2A transfection express the 72-kDa E2A-encoded DBP protein (left panel, lanes 4-14; middle panel, lanes 1-13; right panel, lanes 1-12). In contrast, the only cell line derived from the pcDNAwtE2A transfection did not express the DBP protein (left panel, lane 2). No DBP protein was detected in extract from a cell line derived from the pcDNA3 transfection (left panel, lane 1), which serves as a negative control. Extract from PER.C6 cells transiently transfected with pcDNA3ts125 (left panel, lane 3) served as a positive control for the Western blot procedure. These data confirm that constitutive expression of wtE2A is toxic for cells and that using the ts125 mutant of E2A can circumvent this toxicity.

Figure 32: Suspension growth of PER.C6ts125E2A C5-9.

The tsE2A expressing cell line PER.C6tsE2A.c5-9 was cultured in suspension in serum free Ex-cell<sup>TM</sup>. At the indicated time points, cells were counted in a Burkner cell counter. The results of 8 independent cultures are indicated. PER.C6tsE2A grows well in suspension in serum free Ex-cell<sup>TM</sup> medium.

Figure 33: Growth curve PER.C6 and PER.C6tsE2A.

PER.C6 cells or PER.C6ts125E2A (c8-4) cells were cultured at 37<sup>0</sup>C or 39<sup>0</sup>C, respectively. At day 0, a total of  $1 \times 10^6$  cells was seeded per 25cm<sup>2</sup> tissue culture flask. At the indicated time points, cells were counted. The growth of

PER.C6 cells at 37°C is comparable to the growth of PER.C6ts125E2A c8-4 at 39°C. This shows that constitutive overexpression of ts125E2A has no adverse effect on the growth of cells at the non-permissive temperature of 39°C.

5

Figure 34: Stability of PER.C6ts125E2A.

For several passages, the PER.C6ts125E2A cell line clone 8-4 was cultured at 39°C in medium without G418. Equal amounts of whole-cell extract from different passage numbers were  
10 fractionated by SDS-PAGE on 10% gels. Proteins were transferred onto Immobilon-P membranes and DBP protein was visualized using the  $\alpha$ DBP monoclonal B6 in an ECL detection system. The expression of ts125E2A encoded DBP is stable for at least 16 passages, which is equivalent to approximately  
15 40 cell doublings. No decrease in DBP levels were observed during this culture period, indicating that the expression of ts125E2A is stable, even in the absence of G418 selection pressure.

20 Figure 35: tTA activity in hygromycin resistant PER.C6/tTA (A) and PER/E2A/tTA (B) cells.

Sixteen independent hygromycin resistant PER.C6/tTA cell colonies and 23 independent hygromycin resistant PER/E2A/tTA cell colonies were grown in 10 cm<sup>2</sup> wells to sub-confluency  
25 and transfected with 2  $\mu$ g of pUHC 13-3 (a plasmid that contains the reporter gene luciferase under the control of the 7xtetO promoter). One half of the cultures was maintained in medium containing doxycycline to inhibit the activity of tTA. Cells were harvested at 48 hours after  
30 transfection and luciferase activity was measured. The luciferase activity is indicated in relative light units (RLU) per  $\mu$ g protein.

Table I:

Serotype	Elution [NaCl] mM	VP/ml	CCID50	log <sub>10</sub> VP/CCID50 ratio
1	597	8.66x10 <sup>10</sup>	5.00x10 <sup>7</sup>	3.2
2	574	1.04x10 <sup>12</sup>	3.66x10 <sup>11</sup>	0.4
3	131	1.19x10 <sup>11</sup>	1.28x10 <sup>7</sup>	4.0
4	260	4.84x10 <sup>11</sup>	2.50x10 <sup>8</sup>	3.3
5	533	5.40x10 <sup>11</sup>	1.12x10 <sup>10</sup>	1.7
6	477	1.05x10 <sup>12</sup>	2.14x10 <sup>10</sup>	1.7
7	328	1.68x10 <sup>12</sup>	2.73x10 <sup>9</sup>	2.4
9	379	4.99x10 <sup>11</sup>	3.75x10 <sup>7</sup>	4.1
10	387	8.32x10 <sup>12</sup>	1.12x10 <sup>9</sup>	3.9
12	305	3.64x10 <sup>11</sup>	1.46x10 <sup>7</sup>	4.4
13	231	4.37x10 <sup>12</sup>	7.31x10 <sup>8</sup>	3.8
15	443	5.33x10 <sup>12</sup>	1.25x10 <sup>9</sup>	3.6
16	312	1.75x10 <sup>12</sup>	5.59x10 <sup>8</sup>	3.5
17	478	1.39x10 <sup>12</sup>	1.45x10 <sup>9</sup>	3.0
19	430	8.44x10 <sup>11</sup>	8.55x10 <sup>7</sup>	4.0
20	156	1.41x10 <sup>11</sup>	1.68x10 <sup>7</sup>	3.9
21	437	3.21x10 <sup>11</sup>	1.12x10 <sup>8</sup>	3.5
22	365	1.43x10 <sup>11</sup>	5.59x10 <sup>8</sup>	3.4
23	132	2.33x10 <sup>11</sup>	1.57x10 <sup>7</sup>	4.2
24	405	5.12x10 <sup>12</sup>	4.27x10 <sup>8</sup>	4.1
25	405	7.24x10 <sup>11</sup>	5.59x10 <sup>7</sup>	4.1
26	356	1.13x10 <sup>12</sup>	1.12x10 <sup>8</sup>	4.0
27	342	2.00x10 <sup>12</sup>	1.28x10 <sup>8</sup>	4.2
28	347	2.77x10 <sup>12</sup>	5.00x10 <sup>7</sup>	4.7
29	386	2.78x10 <sup>11</sup>	2.00x10 <sup>7</sup>	4.1
30	409	1.33x10 <sup>12</sup>	5.59x10 <sup>8</sup>	3.4
31	303	8.48x10 <sup>10</sup>	2.19x10 <sup>7</sup>	3.6
33	302	1.02x10 <sup>12</sup>	1.12x10 <sup>7</sup>	5.0
34	425	1.08x10 <sup>12</sup>	1.63x10 <sup>11</sup>	0.8
35	446	3.26x10 <sup>12</sup>	1.25x10 <sup>11</sup>	1.4
36	325	9.26x10 <sup>12</sup>	3.62x10 <sup>9</sup>	3.4
37	257	5.86x10 <sup>12</sup>	2.8x10 <sup>9</sup>	3.3
38	337	3.61x10 <sup>12</sup>	5.59x10 <sup>7</sup>	4.8
39	241	3.34x10 <sup>11</sup>	1.17x10 <sup>7</sup>	4.5
42	370	1.95x10 <sup>12</sup>	1.12x10 <sup>8</sup>	4.2

Continued on next page.

Serotype #	Elution [NaCl] mM	VP/ml	CCID50	log <sub>10</sub> VP/CCID50 ratio
43	284	2.42x10 <sup>12</sup>	1.81x10 <sup>8</sup>	4.1
44	295	8.45x10 <sup>11</sup>	2.00x10 <sup>7</sup>	4.6
45	283	5.20x10 <sup>11</sup>	2.99x10 <sup>7</sup>	4.2
46	282	9.73x10 <sup>12</sup>	2.50x10 <sup>8</sup>	4.6
47	271	5.69x10 <sup>11</sup>	3.42x10 <sup>7</sup>	4.2
48	264	1.68x10 <sup>12</sup>	9.56x10 <sup>8</sup>	3.3
49	332	2.20x10 <sup>12</sup>	8.55x10 <sup>7</sup>	4.4
50	459	7.38x10 <sup>12</sup>	2.80x10 <sup>9</sup>	3.4
51	450	8.41x10 <sup>11</sup>	1.88x10 <sup>8</sup>	3.7

Legend to table I:

- All human adenoviruses used in the neutralisation experiments were produced on PER.C6 cells (ECACC deposit number 96022940) (Fallaux et al., 1998) and purified on CsCl as described in example 1. The NaCl concentration at which the different serotypes eluted from the HPLC column is shown. Virus particles/ml (VP/ml) were calculated from an Ad5 standard.
- The titer in the experiment (CCID50) was determined on PER.C6 cells (ECACC deposit number 96022940) as described in example 1 by titrations performed in parallel with the neutralisation experiment. The CCID50 is shown for the 44 viruses used in this study and reflects the dilution of the virus needed to obtain CPE in 50% of the wells after 5 days. The ratio of VP/CCID50 is depicted in log<sub>10</sub> and is a measurement of the infectivity of the different batches on PER.C6 cells (ECACC deposit number 96022940).

Table II. AdApt35.LacZ viruses escape neutralization by human serum.

Virus	Human serum dilution					
	no serum	10x	50x	250x	1250x	6250x
AdApt5.LacZ moi: 5 VP/cell	100 %	0 %	0 %	1 %	40 %	80 %
AdApt35.LacZ 250 $\mu$ l crude lysate	100 %	100 %	100 %	100 %	100 %	100 %

5

Table III: Percentage of synovial fluid samples containing neutralizing activity (NA) to wt adenoviruses of different serotypes.

10

	% of SF samples with NA (all positives)	% of SF samples with NA (positives at $\geq 64x$ dilution)
Ad5	72	59
Ad26	66	34
Ad34	45	19
Ad35	4	0
Ad48	42	4



Table IV: The numbers of foci obtained with the different E1 expression constructs in BRK transformation experiments.

5 Average #. of foci/dish:

	Construct	1 $\mu$ gr	5 $\mu$ gr
Experiment 1	pIG.E1A.E1 B	nd	60
	pIG.E1A.E1 B	nd	35
	pRSVAd35E1	0	3
	pIG.Ad35.E 1	3	7
Experiment 2	pIG.E1A.E1 B	37	nd
	pIG.Ad35.E 1	nd	2
Experiment 3	pIG.E1A.E1 B	nd	140
	pIG.Ad35.E 1	nd	20
	pIG270	nd	30

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CLAIMS

1. A gene delivery vehicle comprising at least one of the adenovirus serotype 35 elements or a functional equivalent thereof, responsible for avoiding or diminishing neutralising activity against adenoviral elements by the host to which the gene is to be delivered and a gene of interest.
2. Adenovirus serotype 35 or a functional homologue thereof or a chimaeric virus derived therefrom, or a gene delivery vehicle based on said virus its homologue or its chimaera for use as a pharmaceutical.
3. A gene delivery vehicle according to claim 1, whereby said elements comprise adenovirus 35 E3 expression products or the genes encoding them.
4. A gene delivery vehicle according to claim 1 or 3, whereby said elements comprise adenovirus 35 fiber, penton and/or hexon proteins or a gene encoding either.
5. A gene delivery according to any one of claims 1,3, or 4 which is a chimaera of adenovirus 35 with at least one other adenovirus.
6. A gene delivery vehicle according to any one of claims 1,3,4 or 5 which has a different tropism than adenovirus 35.
7. A nucleic acid encoding at least a functional part of a gene delivery vehicle according to any one of claims 1 or 3-6, or a virus, homologue or chimaera thereof according to claim 2.
8. A nucleic acid encoding at least one of the adenovirus serotype 35 elements or a functional equivalent thereof, responsible for avoiding or diminishing neutralising activity against adenoviral elements by the host to which the gene is to be delivered and having a site for introducing a gene of interest therein.
9. A nucleic acid according to claim 7 or 8, further comprising at least one ITR.

10. A nucleic acid according to claim 7, 8 or 9 further comprising a region of nucleotides designed or useable for homologous recombination.
11. At least one set of two nucleic acids comprising a  
5 nucleic acid according to any one of claims 7-10, whereby said set of nucleic acids is capable of a single homologous recombination event with each other, which leads to a nucleic acid encoding a functional gene delivery vehicle.
12. A cell comprising a nucleic acid according to any one of  
10 claims 7-10 or a set of nucleic acids according to claim 11.
13. A cell according to claim 12 which complements the necessary elements for adenoviral replication which are absent from the nucleic acid according to any one of claims 7-10 or a set of nucleic acids according to claim 11.
14. A cell according to claim 12 or 13, which originates  
15 from a PER.C6 cell (ECACC deposit number 96022940).
15. A method for producing a gene delivery vehicle according to claim 1, or 3-6, comprising expressing a nucleic acid according to any one of claims 7-10 in a cell according to  
20 claim 12 or 13 and harvesting the resulting gene delivery vehicle.
16. A method for producing a gene delivery vehicle according to claim 1, or 3-6, comprising culturing a cell according to claim 12 or 13 in a suitable culture medium and harvesting  
25 the resulting gene delivery vehicle.
17. A gene delivery vehicle obtainable by a method according to claims 15 or 16.
18. A gene delivery vehicle according to any one of claims 1,3-6 or 17, which is derived from a chimaera of an  
30 adenovirus and an integrating virus.
19. A gene delivery vehicle according to claim 18, wherein said integrating virus is adeno associated virus.
20. A gene delivery vehicle according to any one of claims 1, 3-6 or 17-19, which has the tropism determining parts of  
35 adenovirus 16 or functional equivalents thereof.



21. A gene delivery vehicle according to anyone of claims 1, 3-6 or 17-20 for use as a pharmaceutical.
22. A pharmaceutical formulation comprising a gene delivery vehicle according to any one of claims 1, 3-6 or 17-20 and a  
5 suitable excipient.
23. A pharmaceutical formulation comprising an adenovirus, a chimaera thereof, or a functional homologue thereof according to claim 2 and a suitable excipient.

**Figure 1:**  
**% of human sera with neutralising capacity for human adenovirus (n=100)**

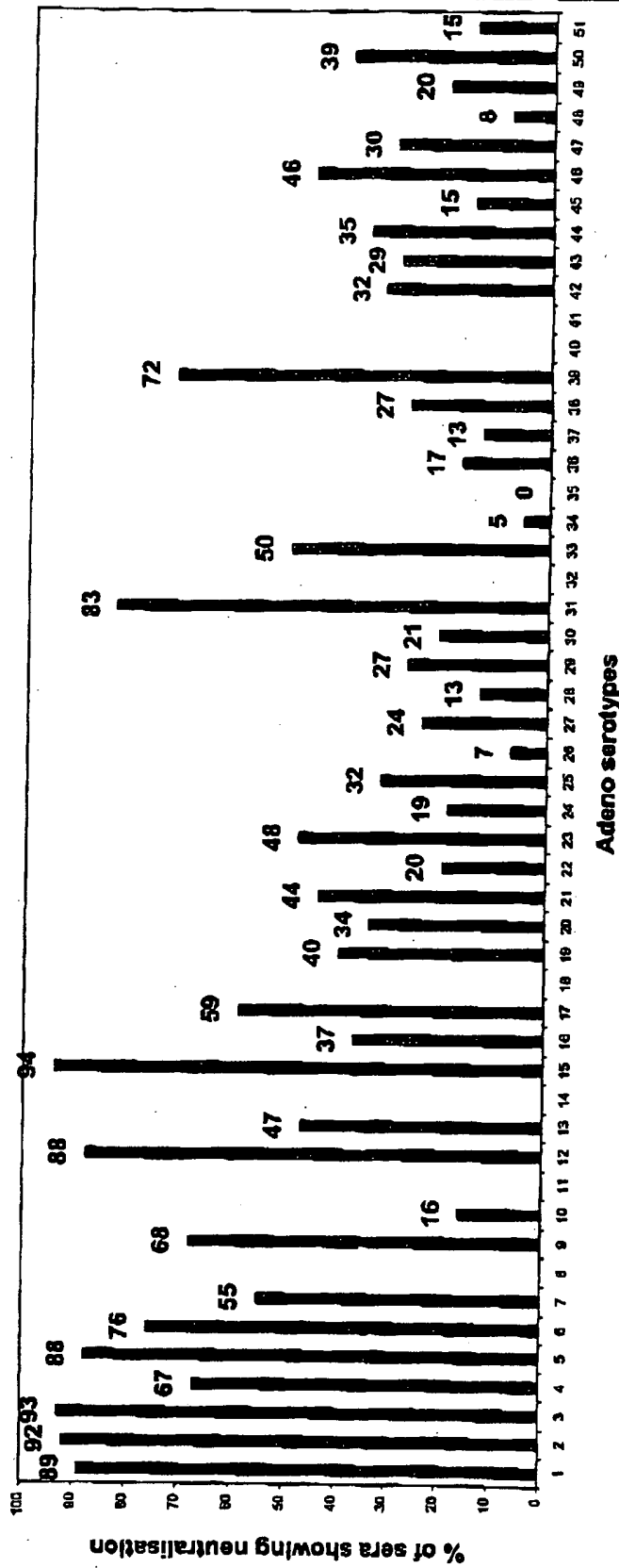


Figure 2

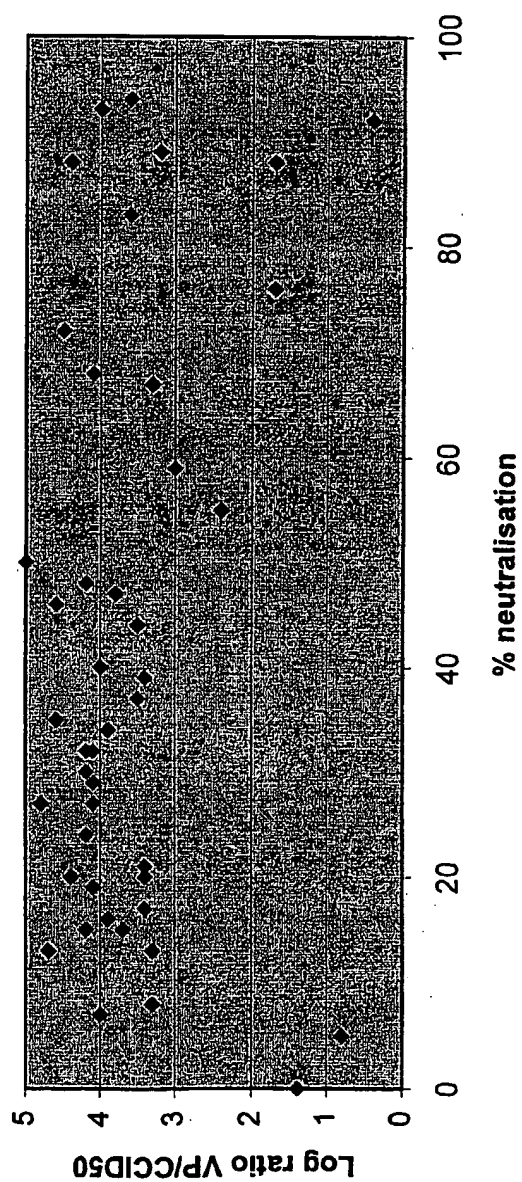
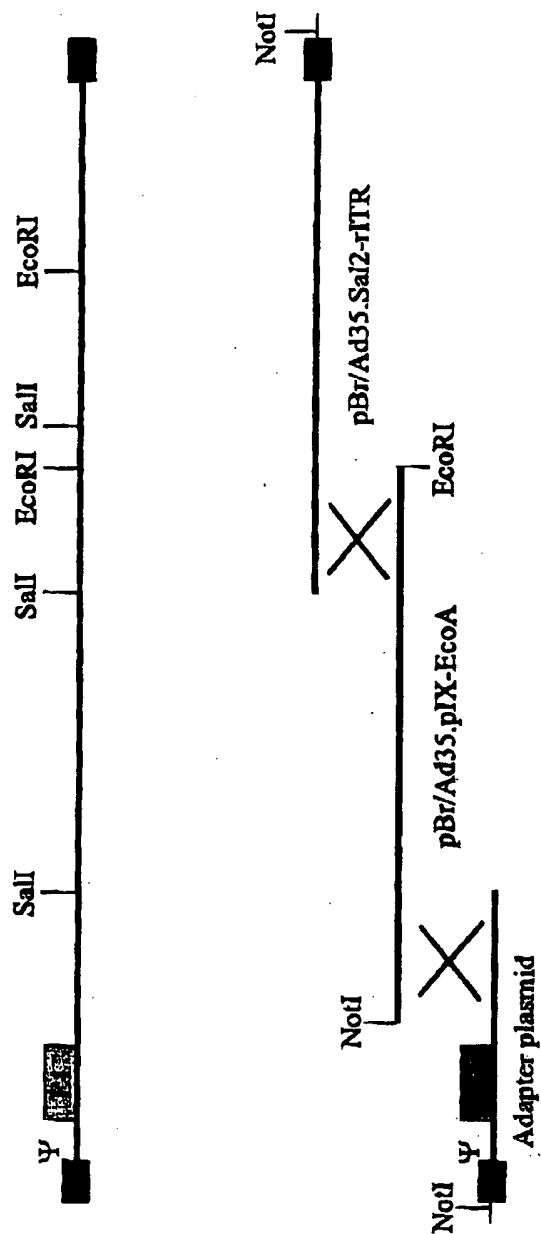
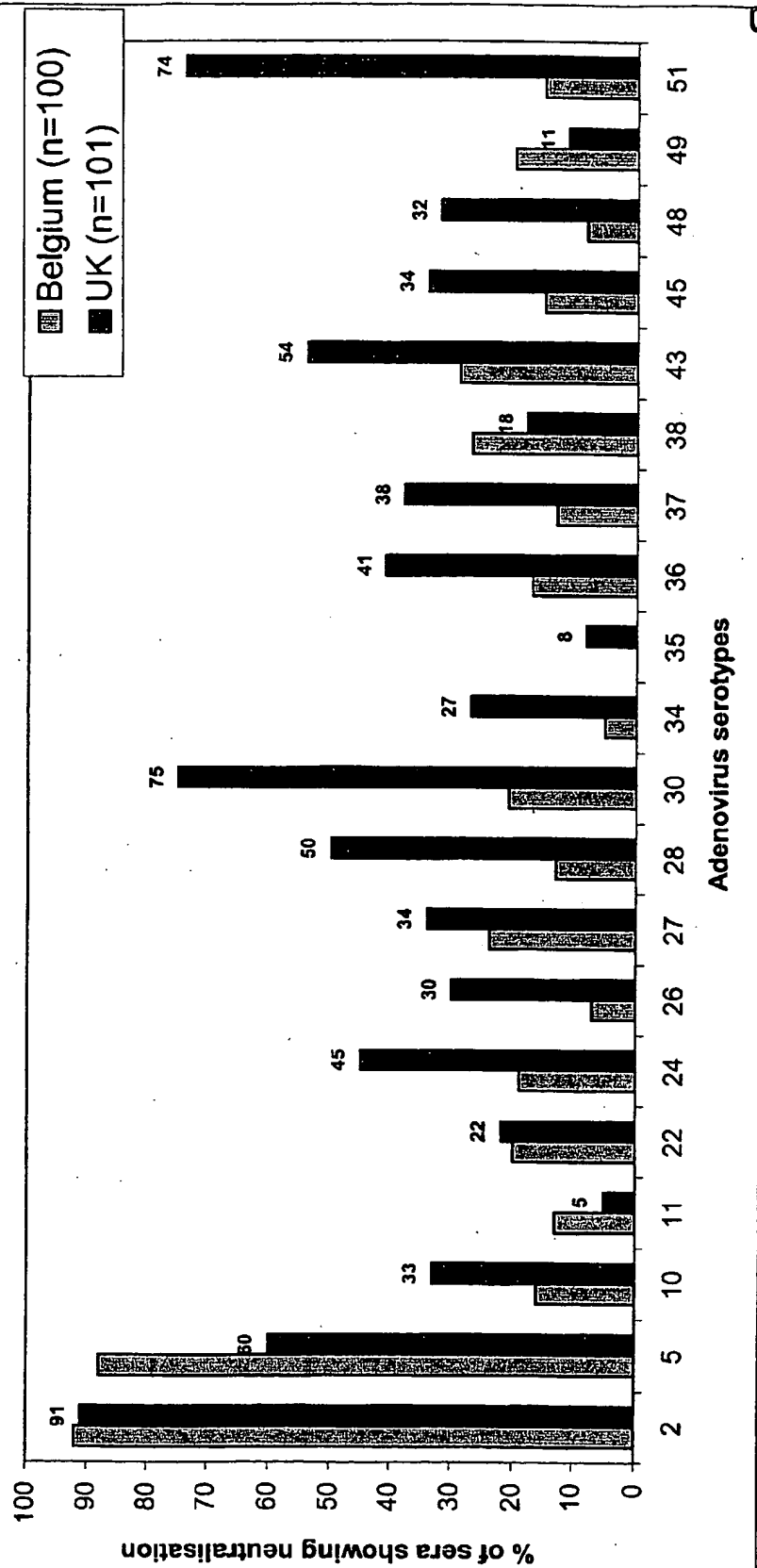


Figure 3: Ad35 plasmid-based system for virus generation



**Figure 4**  
**Neutralisation in human sera**



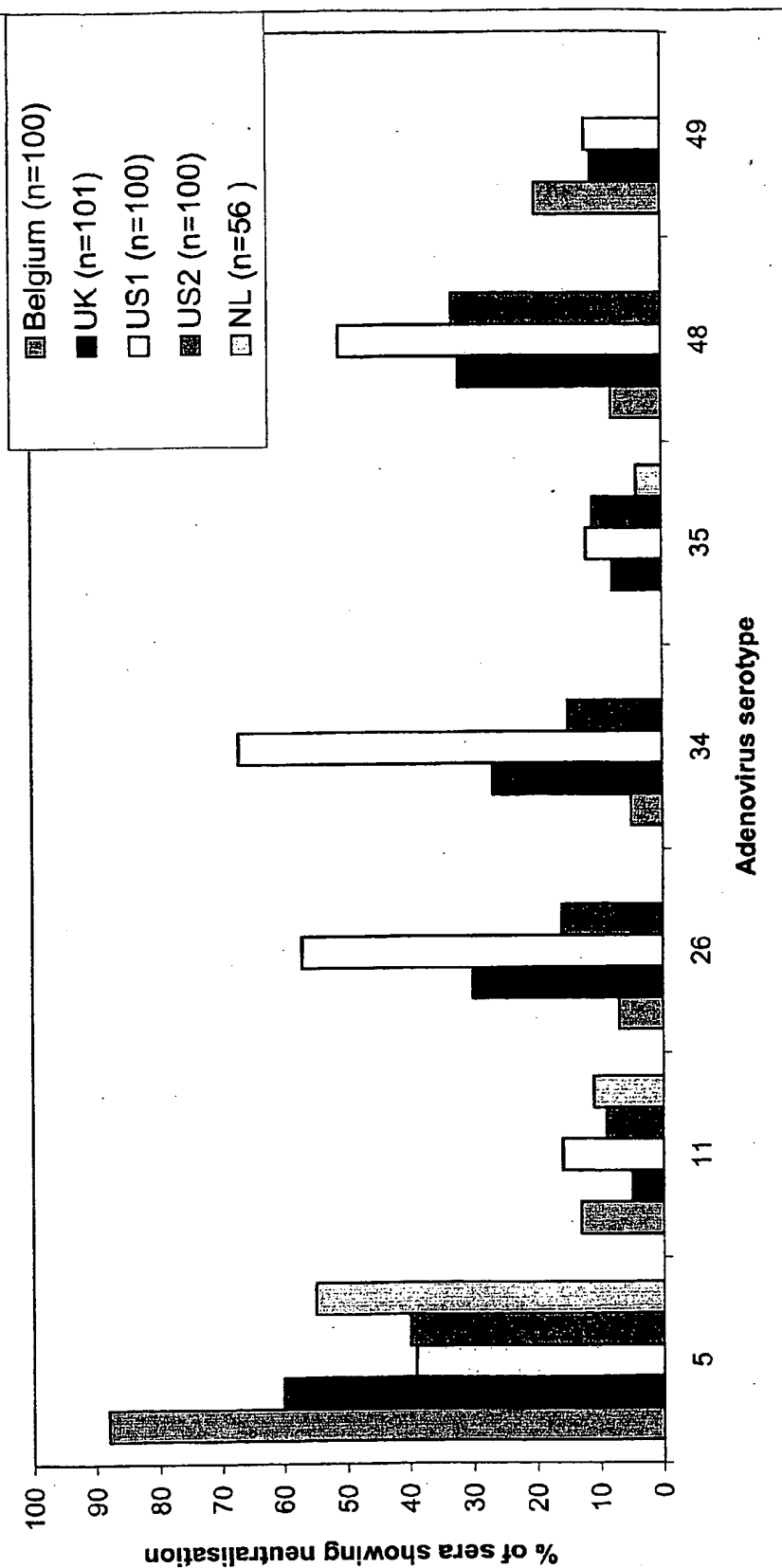
**Figure 5****Neutralisation in human sera from different geographic locations**

Figure 6: Total sequence of Ad35.

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1 CATCATCAAT AATATACCTT ATAGATGGAA TGGTGCCAAT ATGTAAATGA GGTGATTTTA AAAAGTGTGG
71 GCCGTGTGGT GATTGGCTGT GGGGTAAACG GTTAAAAGGG GCGGCGCGGC CGTGGGAAAA TGACGTTTTA
141 TGGGGGTGGA GTTTTTTTGC AAGTTGTGCG GGGAAATGTT ACGCATAAAA AGGCTTCTTT TCTCACGGAA
211 CTACTTAGTT TTCCCACGGT ATTTAACAGG AAATGAGGTA GTTTTGACCG GATGCAAGTG AAAATTGCTG
281 ATTTTTCGCG GAAAACTGAA TGAGGAAGTG TTTTCTGAA TAATGTGGTA TTTATGGCAG GGTGGAGTAT
351 TTGTTACAGG CCAGGTAGAC TTTGACCCAT TACGTGGAGG TTTGATTAC CGTGTTTTTT ACCTGAATTT
421 CCGCGTACCG TGTCAAAGTC TTCTGTTTTT ACGTAGGTGT CAGCTGATCG CTAGGGTATT TATACCTCAG
491 GGTTTGTGTC AAGAGGCCAC TCTTGAGTGC CAGCGAGAAG AGTTTTCTCC TCTGCGCCGG CAGTTTAATA
561 ATAAAAAAT GAGAGATTG CGATTTCTGC CTCAGGAAAT AATCTCTGCT GAGACTGGAA ATGAAATATT
631 GGAGCTTGTG GTGCACGCCC TGATGGGAGA CGATCCGGAG CCACCTGTGC AGCTTTTTGA GCCTCTACG
701 CTTACGGAAC TGTATGATTT AGAGGTAGAG GGATCGGAGG ATTCTAATGA GGAAGCTGTG AATGGCTTTT
771 TTACCGATTG TATGCTTTTA GCTGCTAATG AAGGATTAGA ATTAGATCCG CCTTTGGACA CTTTCAATAC
841 TCCAGGGGTG ATTGTGAAAA GCGGTACAGG TGTAGAAGAA TTACCTGATT TGAGTTCCGT GGACTGTGAT
911 TTGCACTGCT ATGAAGACGG GTTTCCTCCG AGTGATGAGG AGGACCATGA AAAGGAGCAG TCCATGCAGA
981 CTGCAGCGGG TGAGGGAGTG AAGGCTGCCA ATGTTGGTTT TCAGTTGGAT TGCCCGGAGC TTCCTGGACA
1051 TGGCTGTAAG TCTTGTAAT TTCACAGGAA AAATCTGGA GTAAAGGAAC TGTATGTTT GCTTTGTTAT
1121 ATGAGAACGC ACTGCCACTT TATTACAGT AAGTGTGTT AAGTTAAAT TTAAGGAAT ATGCTGTTTT
1191 TCACATGTAT ATTGAGTGTG AGTTTTGTGC TTCTATTAT AGGTCCTGTG TCTGATGCTG ATGAATCACC
1261 ATCTCCTGAT TCTACTACCT CACCTCCTGA TATTCAAGCA CCTGTTCTG TGACGTGCG CAAGCCCAT
1331 CCTGTGAAGC TTAAGCCTGG GAAACGTCCA GCAGTGGAGA AACTTGAGGA CTGTGTACAG GGTGGGGACG
1401 GACCTTTTGA CTTGAGTACA CGGAACCTCA CAAGACAATA AGTGTTCAT ATCCGTGTTT ACTTAAGGTG
1471 ACGTCAATAT TTGTGTGAGA GTGCAATGTA ATAAAAATAT GTTAACTGTT CACTGTTTTT TATGCTTTT
1541 TGGGCGGGGA CTCAGGTATA TAAGTAGAAG CAGACCTGTG TGGTTAGCTC ATAGGAGCTG GCTTTCATCC
1611 ATGGAGGTTT GGGCCATTTT GGAAGACCTT AGGAAGACTA GGCAACTGTT AGAGAGCGCT TCGGACGGAG
1681 TCTCCGTTT TGGAGATTG TGTTGCTGTA GTGAATTAGC TAGGGTAGTT TTTAGGATAA AACAGGACTA
1751 TAAACAAGAA TTTGAAAAGT TGTGGTAGA TGTCGAGGA CTTTTTGAAG CTCTTAATTT GGGCCATCAG
1821 GTTCACTTTA AAGAAAAAGT TTTATCAGTT TTAGACTTTT CAACCCAGG TAGAACTGCT GCTGCTGTGG
1891 CTTTTCTTAC TTTTATATTA GATAAATGGA TCCCGCAGAC TCATTTTCAGC AGGGGATACG TTTTGGATTT
1961 CATAGCCACA GCATTGTGGA GAACATGGAA GGTTCGCAAG ATGAGGACAA TCTTAGGTTA CTGGCCAGTG
2031 CAGCCTTTGG GATGAGCGGG AATCCTGAGG CATCCACCGG TCATGCCAGC GGTTCCTGGA GAGGAACAAG
2101 AAGAGGACAA CCCGAGAGCC GGCCTGGACC CTCCAGTGGG GGAGGCGGAG TAGCTGACTT GTCTCCTGAA
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2311 GCATGAGGTT TGAAGAGAGG GAAGGGATGA AGTTTCTGTA TTGCAGGAGA AATATTCACT GGTTCCTGAG GGAAGAGTG
2381 AAAACATGTT GGTGGAGGCC AGAGGATGAT TGGGCGGTGG CCATTAAAAA TTATGCCAAG ATAGCTTTGA
2451 GGCCTGATAA ACAGTATAAG ATCAGTAGAC GGATTAATAT CCGGAATGCT TGTACATAT CTGGAATGG
2521 GGTGAGGTG GTAATAGATA CTCAAGACAA GACAGTTAAT AGATGCTGCA TGATGGATAT GTGGCCTGGA
2591 GTAGTCGGTA TGAAGCAGT CACTTTTGTG AATGTTAAGT TTAGGGGAGA TGGTTAAT TGGTATGTG
2661 TTATGGCCAA TACCAAACCT ATATTGCATG GTTGTAGCTT TTTTGGTTT AACAATACCT GTGTAGATGC
2731 CTGGGGACAG GTTAGTGTAC GGGGGTGTAG TTTCTATGCG TGTGGATTG CCACAGCTGG CAGAACCAAG
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3081 CCAAGTGCAC CATGCATGCA GGTGGGCGTA GAGGAATGTT TATGCCTTAC CAGTGTAACA TGAATCATGT
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4061 GAATGTTTAG ATACATGGG CTTAGGCCGT CTTTGGGGTG GAGATAGCTC CATTGAAGGG ATTCATGCTC
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Figure 6, contd.

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8051 GCCGAGGCTT GCGGATTGTA TCTCGTGCTC TTCTATATTC GCTGTATCGG CCTGTTTCATC TTCTGTTTCG
8121 ATGGTGGTCA TGCTGACGAG CCCCCGCGG AGGCAAGTCC AGACCTCGG CCGGGAGGGG CCGAGCTGAA
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8261 CAGAAGATTA ACTTGCATGA TCTTTTCCAG GCGCTGCGGG AGGTTGAGT GGTACTTGAT TTCCACAGGT
8331 TCGTTTTGTAG AGACGTCAT GGTTCGAGG GTTCCGTTG CTTTGGCGC CACTACCGTA CCTTTGTTTT
8401 TTCTTTTGAT CGGTGGTGGC TCTCTTGCTT CTTGCATGCT CAGAAGCGGT GACGGGGACG CCGCGCGGGC
8471 GGCAGCGGTT GTTCCGACC CCGGGGCATG GCTGGTAGTG GCACGTGCGG GCCCGCACG GGCAGGTTCT
8541 GGTATTGCGC TCTGAGAAGA CTTGCGTGG CACACCAGCG TCGATTGACG TCTTGATCT GAGCTCTCTG
8611 GGTGAAAGCT ACCGGCCCG TGAGCTTGAA CCTGAAAGAG AGTTCAACAG AATCAATTTT GATTCGTTTA
8681 ACGGCAGCTT GTCTCAGTAT TTCTTGACG TCACCAGAGT TGTCTGGTA GCGGATCTCC GCCATGAACT
8751 GCTCGATTTT TTCCTCTGA AGATCTCCGC GACCCGCTCT TCGACGGTG GCCGCGAGGT CATTGGAGAT

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Figure 6, contd.

8821	ACGGCCCATG	AGTTGGGAGA	ATGCATTTCAT	GCCCCGCTCG	TTCCAGACGC	GGCTGTAAAC	CACGGCCCCC
8891	TCGGAGTCTC	TTGCGCGCAT	CACCACCTGA	GCGAGGTTAA	GCTCCACGTG	TCTGGTGAAG	ACCGCATAGT
8961	TGCATAGGCG	CTGAAAAAGG	TAGTTGAGTG	TGGTGGCAAT	GTGTTGCGCG	ACGAAGAAAT	ACATGATCCA
9031	TCGTCTCAGC	GGCATTTCGC	TAACATCCGC	CAGAGCTTCC	AAGCGCTCCA	TGGCCTCGTA	GAAGTCCACG
9101	GCAAAATTAA	AAAACCTGGG	GTTTCGCGCG	GACACGGTCA	ATTCTCTCTC	GAGAAGACGG	ATGAGTTCGG
9171	CTATGGTGGC	CCGTACTTCG	CGTTCGAAGG	CTCCCGGGAT	CTCTTCTTCC	TCTTCTATCT	CTTCTTCCAC
9241	TAACATCTCT	TCTTCGTCTT	CAGGCGGGGG	CGGAGGGGGC	ACGCGGCGAC	GTCGACGGCG	CACGGGCAAA
9311	CGGTGATGA	ATCGTTCAAT	GACCTCTCCG	CGGCGGCGGC	GCATGGTTTC	AGTGACGGCG	CGGCCCTTCT
9381	CGCGCGGTG	CAGAGTAAAA	ACACCGCCGC	GCATCTCCTT	AAAGTGGTGA	CTGGGAGGTT	TCCGTTTGG
9451	GAGGGAGAGG	GCGCTGATTA	TACATTTTAT	TAATTGGCCC	GTAGGGACTG	CGCGCAGAGA	TCTGATCGTG
9521	TCAAGATCCA	CGGGATCTGA	AAACCTTTCG	ACGAAAGCGT	CTAACCAGTC	ACAGTCACAA	GGTAGGCTGA
9591	TACGGCTCTC	TTGTGGGCGG	GGGTGGTTAT	GTGTTCCGGT	TGGGTCTTCT	GTTTCTTCTT	CATCTCGGGA
9661	AGGTGAGACG	TGATGCTGG	GAATGAATTT	AAAGTAGGCA	GTTCTAAGAC	GGCGGATGGT	GGCGAGGAGC
9731	ACCAGGTCTT	TGGGTCCGGC	TTGCTGGATA	CGCAGGCGAT	TGGCCATTCC	CCAAGCATT	TCCTGACATC
9801	TAGCAAGATC	TTTGTAGTAG	TCTTGCATGA	GCCGTTCTAC	GGGCACTTCT	TCCTCACCCG	TTCTGCCATG
9871	CATACGTGTG	AGTCCAAATC	CGCGCATTTG	TTGTACCAGT	GCCAAGTCAG	CTACGACTCT	TTCGGCGAGG
9941	ATGGCTTGCT	GTAACCTGGT	AAGGGTGGGT	TGAAAGTCAT	CAAAATCCAC	AAAGCGGTGG	TAAGCCCTCT
10011	TATTAATGGT	GTAAGCACAG	TTGGCCATGA	CTGACCAGTT	AACTGTCTGG	TGACCAGGGC	GCACGAGCTC
10081	GGTGTATTTA	AGGCGCGAAT	AGGCGCGGGT	GTCAAAGATG	TAATCGTTGC	AGGTGCGCAC	CAGATACTGG
10151	TACCCATATA	GAAATATGCG	CGGTGGTTGG	CGGTAGAGAG	GCCATCGTTC	TGTAGCTGGA	CGCGCAGGGG
10221	CGAGGCTCTT	CAACATAAGG	CGGTGATAGC	CGTAGATGTA	CCTGGACATC	CAGGTGATTC	TGCGCGGGT
10291	AGTAGAAGCC	CGAGGAAACT	CGCGTACGCG	GTTCCAAATG	TTGCGTAGCG	GCATGAAGTA	GTTTATTGTA
10361	GGCACGGTTT	GACCACTGAG	GCGCGCGCAG	TCATTGATGC	TCTATAGACA	CGGAGAAAAT	GAAAGCGTTC
10431	AGCGACTCGA	CTCCGTAGCC	TGGAGGAACG	TGAACGGGTT	GGGTGCGGGT	GTACCCCGGT	TGAGAGACTG
10501	TACTCGAGCC	GCCGCGAGCC	GCGGCTAACG	TGGTATTGGC	ACTCCCGTCT	CGACCCAGCC	TACAAAAATC
10571	CAGGATACGG	AATCGAGTCG	TTTTGCTGGT	TTCCGAATGG	CAGGGAAGTG	AGTCCTATTT	TTTTTTTTTT
10641	TTTGCCGCTC	AGATGCATCC	CGTGCTGCGA	CAGATGCGCC	CCCAACAACA	GCCCCCTCTG	CAGCAGCAGC
10711	AGCAGCAACC	ACAAAAGGCT	GTCCCTGCAA	CTACTGCAAC	TGCCGCGGTG	AGCGGTGCGG	GACAGCCCGC
10781	CTATGATCTG	GACTTGGAA	AGGGCGAAGG	ACTGGCACTG	CTAGGTGCGC	CTTCGCCCGA	GCGGCATCCG
10851	CGAGTTCAAC	TGAAAAAAGA	TTCTCGCGAG	GCGTATGTGC	CCCAACAGAA	CCTATTTAGA	GACAGAAGCG
10921	GCGAGGAGCC	GGAGGAGATG	CGAGCTTCCC	GCTTTAACGC	GGGTGCTGAG	CTGCGTCACG	GTTTGGACCG
10991	AAGACGAGTG	TTGCGAGACG	AGGATTTGGA	AGTTGATGAA	GTGACAGGGA	TCAGTCCTGC	CAGGGCACAC
11061	GTGGCTGCG	CCAACCTTGT	ATCGGCTTAC	GAGCAGACAG	TAAAGGAAGA	GCGTAACCTT	CAAAAGTCTT
11131	TTAATAATCA	TGTGCGAACC	CTGATTGCCC	GCGAAGAAGT	TACCTTTGGT	TFGATGCATT	TGTGGGATTT
11201	GATGGAAGCT	ATCATTTCAG	ACCCTACTAG	CAAACCTCTG	ACCGCCAGC	TGTTTCTGGT	GGTGCAACAC
11271	AGCAGAGACA	ATGAGGCTTT	CAGAGAGGCG	CTGCTGAACA	TCACCGAACC	CGAGGGGAGA	TGGTTGTATG
11341	ATCTTATCAA	CATTCTACAG	AGTATCATAG	TGCAGGAGCG	GAGCCTGGGC	CTGGCCGAGA	AGGTAGCTGC
11411	CATCAATTAC	TCGGTTTTGA	GCTTGGGAAA	ATATTACGTT	CGCAAAATCT	ACAAGACTCC	ATACGTTCCC
11481	ATAGACAAGG	AGGTGAAGAT	AGATGGGTTT	TACATGCGCA	TGACGCTCAA	GGTCTTGACC	CTGAGCGATG
11551	ATCTTGGGGT	GTATCGCAAT	GACAGAAATG	ATCGCGCGGT	TAGCGCCAGC	AGGAGGCGCG	AGTTAAGCGA
11621	CAGGGAACCT	ATGCACAGTT	TGCAAGAGAG	TCTGACTGGA	GCTGGAACCG	AGGGTGAGAA	TTACTTGCAG
11691	ATGGGAGCTG	ACTTGCAGTG	GCAGGCTAGT	GACAGGGGTC	TGAGCGCCGC	GACGGCAGGA	CTGAGGCTTC
11761	CTTACATAGA	AGAGGCGGAT	GAAGGCGAGG	AGGAAGAGGG	CGAGTACTTG	GAAGACTGAT	GGCACAACCC
11831	GTGTTTTTTG	CTAGATGGAA	CAGCAAGCAC	CGGATCCCGC	AATGCGGGCG	GCGCTGCAGA	GCCAGCCGTC
11901	CGGCATTAAC	TCCTCGGACG	ATTGGACCCA	GGCCATGCAA	CGTATCATGG	CGTTGACGAC	TCGCAACCCC
11971	GAAGCCTTTA	GACAGCAACC	CCAGGCCAAC	CGTCTATCGG	CCATCATGGA	AGCTGTAGTG	CCTTCCCGAT
12041	CTAATCCCAC	TCATGAGAAG	GTCCTGGCCA	TCGTGAACGC	GTTGGTGGAG	AACAAAGCTA	TTCGTCCAGA
12111	TGAGGCCGGA	CTGGTATACA	ACGCTCTCTT	AGAACGCGTG	GCTCGCTACA	ACAGTAGCAA	TGTGCAAAAC
12181	AATTTGGACC	GTATGATAAC	AGATGTACGC	GAAGCCGTGT	CTCAGCGCGA	AAGBTTCCAG	CGTGATGCCA
12251	ACCTGGGTTT	GCTGGTGGCG	TTAAATGCTT	TCTTGAGTAC	TCAGCCTGCT	AATGTGCCCG	GTTGTCAACA
12321	GGATTATACT	AACTTTTTTA	GTGCTTTGAG	ACTGATGGTA	TCAGAAGTAC	CTCAGAGCGA	AGTGTATCAG
12391	TCCGGTCTCG	ATTACTTCTT	TCAGACTAGC	AGACAGGGCT	TGCAGACGGT	AAATCTGAGC	CAAGCTTTTA
12461	AAAACCTTAA	AGGTTTGTGG	GGAGTGCATG	CCCCGGTAGG	AGAAAGAGCA	ACCGTGCTTA	GCTTGTTAAC
12531	TCCGAATCCC	CGCCTGTTAT	TACTGTTGGT	AGCTCCTTTT	ACCGACAGCG	GTAGCATCGA	CCGTAATTCC
12601	TATTTGGGTT	ACCTACTAAA	CCTGTATCGC	GAAGCCATAG	GGCAAAGTCA	GGTGGACGAG	CAGACCTATC
12671	AAGAAATTAC	CCAAGTCAGT	CGCGCTTTGG	GACAGGAAGA	CACTGGCAGT	TTGGAAGCCA	CTCTGAACCT
12741	CTTGCTTACC	AATCGGTCTC	AAAAGATCCC	TCCTCAATAT	GCTCTTACTG	CGGAGGAGGA	GAGGATCTTT
12811	AGATATGTGC	AGCAGAGCGT	GGGATTGTTT	CTGATGCAAG	AGGGGGCAAC	TCCGACTGCA	GCCTGGGACA
12881	TGACAGCGCG	AAATATGGAG	CCCAGCATGT	ATGCCAGTAA	CCGACCTTTC	ATTAACAAC	CTTCCGACTA
12951	CTTGACAGAG	GCTGCCGCTA	TGAACCTCTG	TTATTTCAAC	AATGCCATCT	TAAACCCGCA	CTGGCTGCCC
13021	CCACCTGGTT	TCTACACGGG	CGAATATGAC	ATGCCCGACC	CTAATGACGG	ATTTCTGTGG	GACGACGTGG
13091	ACAGCGATGT	TTTTTACCTT	CTTTCTGATC	ATCGCACGTG	GAAAAAGGAA	GGCGGTGATA	GAATGCATTC
13161	TTCTGCTATC	CTGTCCGGGG	TCATGCTGTC	TACCGCGGCT	GAGCCCGAGT	CTGCAAGTCC	TTTTCTTAGT
13231	CTACCCCTTT	CTCTACACAG	TGTACGTAGC	AGCGAAGTGG	GTAGAATAAG	TCGCCCGAGT	TTAATGGGCG

Figure 6, contd.

13301	AAGAGGAGTA	CCTAAACGAT	TCCTTGCTCA	GACCGGCAAG	AGAAAAAAT	TTCCCAAACA	ATGGAATAGA
13371	AAGTTTGGTG	GATAAAATGA	GATAGATGGAA	GACTTATGCT	CAGGATCACA	GAGACGAGCC	TGGGATCATG
13441	GGGACTACAA	GATAGAGCGAG	CCGTAGACGC	CAGCGCCATG	ACAGACAGAG	GGGTCTTGTG	TGGGACGATG
13511	AGGATTCGGC	CGATGATAGC	AGCGTGTGG	ACTTGGGTGG	GAGAGGAAGG	GGCAACCCGT	TTGCTCATTT
13581	GCGCCCTCGC	TTGGGTGGTA	TGTTGTGAAA	AAAAATAAAA	AAGAAAAACT	CACCAAGGCC	ATGGCGACGA
13651	GCGTACGTTT	GTTCTTCTTT	ATTATCTGTG	TCTAGTATAA	TGAGGCGAGT	CGTGCTAGGC	GGAGCGGTGG
13721	TGTATCCGGA	GGGTCTCTCT	CCTTCGTACG	AGAGCGTGAT	GCAGCAGCAG	CAGGCGACGG	CGGTGATGCA
13791	ATCCCCACTG	GAGGCTCCCT	TTGTGCCTCC	GCGATACCTG	GCACCTACGG	AGGGCAGAAA	CAGCATTCTG
13861	TACTCGGAAC	TGGCACCTCA	GTACGATACC	ACCAGGTTGT	ATCTGGTGGG	CAACAAGTCG	GCGGACATTG
13931	CTTCTCTGAA	CTATCAGAAT	GACCACAGCA	ACTTCTTGAC	CACGGTGGTG	CAGAACAATG	ACTTTACCCC
14001	TACGGAAGCC	AGCACCCAGA	CCATTAACCT	TGATGAACGA	TCGCGGTGGG	GCGGTACAGT	AAAGACCATC
14071	ATGCATACTA	ACATGCCAAA	CGTGAACGAG	TATATGTTTA	GTAACAAGTT	CAAAGCGCGT	GTGATGGTGT
14141	CCAGAAAAAC	TCCCGACGGT	GCTGCAGTTG	GGGATACITTA	TGATCACAAG	CAGGATATTT	TGGAATATGA
14211	GTGGTTCGAG	TTTACTTTGC	CAGAAGGCAA	CTTTTCAGTT	ACTATGACTA	TTGATTTGAT	GAACAATGCC
14281	ATCATAGATA	ATTACTTGAA	AGTGGGTAGA	CAGAATGGAG	TGCTTGAAAG	TGACATTGGT	GTTAAGTTTCG
14351	ACACCAGGAA	CTTCAAGCTG	GGATGGGATC	CCGAAACCAA	GTTGATCATG	CCTGGAGTGT	ATACGTATGA
14421	AGCTTCCAT	TGGTACATTG	TCTTACTGCC	TGGTCCGGA	GTGGATTTTA	CCGAGAGTCG	TTTGAGCAAC
14491	CTTCTTGGTA	TCAGAAAAAA	ACAGCCATTT	CAAGAGGGTT	TTAAGATTTT	GTATGAAGAT	TTAGAAGGTG
14561	GTAATATTCC	GGCCCTCTTG	GATGTAGATG	CCTATGAGAA	CAGTAAGAAA	GAACAAAAAG	CCAAAAATAGA
14631	AGCTGCTACA	GCTGCTGCAG	AAGCTAAGGC	AAACATGAT	GCCAGCGACT	CTACAAGGGT	TGCTAACGCT
14701	GGAGAGGTC	GAGGAGACAA	TTTTGCGTCC	ACACCTGTTC	CGACTGCAGA	ATCATTATTG	GCCGATGTGT
14771	CTGAAGGAAC	GGACGTGAAA	CTCACTATTC	AACTGTGAGA	AAAAGATAGT	AAGAATAGAA	GCTATAATGT
14841	GTTGGAAGAC	AAAATCAACA	CAGCCTATCG	CAGTTGGTAT	CTTTCGTACA	ATTATGGCGA	TCCCGAAAAA
14911	GGAGTGCCTT	CCTGGACATT	GCTCACCACC	TCAGATGTCA	CCTGCGGAGC	AGAGCAGGTT	TACTGGTCCG
14981	TTCCAGACAT	GATGAAGGAT	CCTGTCACTT	TCCGCTCCAC	TAGACAAGTC	AGTAACCTAC	CTGTGGTGGG
15051	TGCAGAGCTT	ATGCCCGTCT	TCTCAAAGAG	CTTCTACAAC	GAACAAGCTG	TGTACTCCCA	GCAGCTCCGC
15121	CAGTCCACCT	CGCTTACGCA	CGTCTTCAAC	CGCTTTCCTG	AGAACCAGAT	TTAATCCGT	CCGCCGGCGC
15191	CCACCATTAC	CACCGTCAGT	GAAAACGTTT	CTGCTCTCAC	AGATCACGGG	ACCCTGCCGT	TGCGCAGCAG
15261	TATCCGGGGA	GTCCAACGTG	TGACCGTTAC	TGACGCGAGA	CGCCGCACCT	GTCCTCATCT	ATTACTAGCA
15331	CTGGGCTAG	TGCCACCGCG	CGTCTTTTCA	AGCGGCACCT	TCTAAAAAAA	AAAAATGTCC	ATTCTTATCT
15401	CGCCCAAGTA	TAACACCGGT	TGGGGTCTGC	GCGCTCCAAG	CAAGATGTAC	GGAGGCGCAC	GCAAACGTTT
15471	TACCCAACAT	CCCGTGCCTG	TTCGCGGACA	TTTTCGCGCT	CCATGGGGTG	CCCTCAAGGG	CCGCACTCCG
15541	GTTTCAACCA	CCGTGATGTA	TGTAATCGAT	CAGGTGGTTG	CCGACGCCCC	TAATTATAT	CCTACTGCGC
15611	CTACAGTCT	TGTGGATGCA	GTTATTGACA	GAGTGTGGC	TGACGCTCGC	AACATGCTC	GACGTAAAGG
15681	CCGGCGAAGG	CGCATTGCCA	GACGCCACCG	AGCTACCACT	GCCATGCGAG	CCGCAAGAGC	TCTGCTACGA
15751	AGAGCTAGAC	GCGTGGGGCG	AAGAGCCATG	CTTAGGGCGG	CCAGACGTGC	AGCTTCGGGC	GCCAGCGCCG
15821	GCAGGTCCCG	CAGGCAAGCA	GCCGCTGTGC	CAGCGCGCAG	TATTGCCGAC	ATGGCCCAAT	CGCGAAGAGG
15891	CAATGTATAC	TGGGTGCGTG	ACGCTGCCAT	CGGTCAACGT	GTACCCGTGC	GCACCCGTCC	CCCTCAGACT
15961	TAGAAGATAC	TGAGCAGTCT	CCGATGTTGT	GTCCAGCGGG	CGAGGATGTC	CAAGCGCAAA	TACAAGGAAG
16031	AAATGTGTC	GGTTATCGCA	CCTGAAGTCT	ACGGCCAACC	GTTGAAGGAT	GAACAAAAAC	CCCGCAAAAT
16101	CAAGCGGGTT	AAAAAGGACA	AAAAAGAGCA	GGAAGATGGC	GATGATGGGG	TGGCGGAGTT	TGTGCGCGAG
16171	TTTGGCCAC	GCGCAGCGGT	GCAATGGCGT	GGAGCGCAAG	TTCGACATGT	GTTGAGACCT	GGAATCTCGG
16241	TGGTCTTTAC	ACCCGGCGAG	CGTTCAAGCG	CTACTTTTAA	GCGTTTCTAT	GATGAGGTGT	ACGGGGATGA
16311	TGATATTCTT	GAGCAGGCGG	CTGACCGATT	AGGCGAGTTT	GCTTATGGCA	AGCGTAGTAG	AATAACTTCC
16381	AAGGATGAGA	CAGTGTCAAT	ACCCTTGGAT	CATGGAATC	CCACCCCTAG	TCTTAAACCG	GTCACCTTGC
16451	AGCAAGTGTT	ACCCGTAAC	CCGCGAACAG	GTGTTAAACG	CGAAGGTGAA	GATTTGTATC	CCACTATGCA
16521	ACTGATGGTA	CCCAAACGCC	AGAAGTTGGA	GGACGTTTTG	GAGAAAGTAA	AAGTGGATCC	AGATATTCAA
16591	CCTGAGGTTA	AAGTGAGACC	CATTAAGCAG	GTAGCGCCTG	GTCTGGGGGT	ACAAACTGTA	GACATTAAGA
16661	TTCCCACTGA	AAGTATGGAA	GTGCAAACTG	AACCCGCAAA	GCCTACTGCC	ACCTCCACTG	AAGTGCAAA
16731	GGATCCATGG	ATGCCCATGC	CTATTACAAT	TGACGCGCGC	GGTCCCACTC	GAAGATCCCG	ACGAAAGTAC
16801	GGTCCAGCAA	GTCTGTTGAT	GCCCAATTAT	TTGTGACACC	CATCTATTAT	TCCTACTCCT	GGTTACCGAG
16871	GCACTCGCTA	CTATCGCAGC	CGAAACAGTA	CCTCCCGCCG	TGCGCGCAAG	ACACCTGCAA	ATCGCAGTCG
16941	TGCGCGTAGA	CGCACAAAGCA	AACCGACTCC	CGGCGCCCTG	GTGCGGCAAG	TGTACCGCAA	TGGTAGTGCG
17011	GAACCTTTGA	CACTGCCGCG	TGCGCGTTAC	CATCCGAGTA	TCATCACTTA	ATCAATGTTG	CCGCTGCCTC
17081	CTGCTGACAT	TGCGCCTCAC	TTGTGCGCTT	CGCGTTCCCA	TCACTGGTTA	CCGAGGAAGA	AACCTGCGCC
17151	GTAGAAGAGG	GATGTTGGGA	CGCGGAATGC	GACGCTACAG	GCGACGGCGT	GCTATCCGCA	AGCAATTGCG
17221	GGGTGGTTTT	TTACCAGCCT	TAATTCCAAT	TATCGCTGCT	GCAATTGGCG	CGATACCAGG	CATAGCTTCC
17291	TGTGGCGGTT	AGGCGTCCGA	ACGACATTGA	CATTGGAATA	AAAACGTATA	AATAAAAAAA	AATACAATGG
17361	ACTGCTGAC	TCTGTGCTCT	GTGACTATGT	TTTCTTAGAG	ATGGAAGACA	TCAATTTTTT	ATCTTGGCT
17431	CCGCGACACG	GCACGAAGCC	GTACATGGGC	ACCTGGAGCG	ACATCGGCAC	GAGCCAACCT	AACGGGGGCG
17501	CCTTCAATTG	GAGCAGTATC	TGGAGCGGGC	TTAAAAATTT	TGGCTCAACC	ATAAAAAACAT	ACGGGAACAA
17571	AGCTTGGAAC	AGCAGTACAG	GACAGGCGCT	TAGAAAAATA	CTTAAAGACC	AGAACTTCCA	ACAAAAGTAA
17641	GTCGATGGGA	TAGCTTCCGG	CATCAATGGA	TGGGTAGATT	TGGCTAACCA	GGGTGTGCAG	AAAAAGATAA
17711	ACAGTCGTTT	GGACCCGCCG	CCAGCAACCC	CAGGTGAAAT	GCAAGTGGAG	GAAGAAATTC	CTCCGCCAGA

Figure 6, contd.

17781	AAAACGAGGC	GACAAGCGTC	CGCGTCCCGA	TTTGAAGAG	ACGCTGGTGA	CGCGCGTAGA	TGAACCGCCT
17851	TCTTATGAGG	AAGCAACGAA	GCTTGAATG	CCCACCACTA	GACCGATAGC	CCCAATGGCC	ACCGGGGTGA
17921	TGAAACCTTC	TCAGTTGCAT	CGACCCGTCA	CCTTGGATTT	GCCCCCTCCC	CCTGCTGCTA	CTGCTGTACC
17991	CGCTTCTAAG	CCTGTGCGTG	CCCCGAAACC	AGTCGCCGTA	GCCAGGTCAC	GTCCCGGGGG	CGCTCCTCGT
18061	CCAAATGCGC	ACTGGCAAAA	TACTCTGAAC	AGCATCGTGG	GTCTAGGCGT	GCAAAGTGTA	AAACGCCGTC
18131	GCTGCTTTTA	ATTAATATG	GAGTAGCGCT	TAACCTGCCT	ATCTGTGTAT	ATGTGTCAAT	ACACGCCGTC
18201	ACAGCAGCAG	AGGAAAAAAG	GAAGAGGTG	TGCGTCGACG	CTGAGTTACT	TTCAAGATGG	CCACCCCATC
18271	GATGCTGCCC	CAATGGGCAT	ACATGCACAT	CGCCGGACAG	GATGCTTCGG	AGTACCTGAG	TCCGGGTCTG
18341	GTGCAGTTGG	CCCGCGCCAC	AGACACCTAC	TTCAATCTGG	GAAATAAGTT	TAGAAATCCC	ACCGTAGCGC
18411	CGACCCACGA	TGTGACCACC	GACCGTAGCC	AGCGGCTCAT	GTTGCGCTTC	GTGCCCCTTG	ACCGGGAGGA
18481	CAATACATAC	TCTTACAAAG	TGCGGTACAC	CCTGGCCGTG	GGCGACAACA	GAGTGCTGSA	TATGGCCAGC
18551	ACGTTCTTTG	ACATTAGGGG	CGTGTGGGAC	AGAGGTCCCA	GTTTCAAACC	CTATTCTGGT	ACGGCTTACA
18621	ACTCTCTGGC	TCTTAAAGGC	GCTCCAAATG	CTTCAATCTG	GATTGCAAAA	GCGGTACCAA	CTGCAGCAGC
18691	CGCAGGCAAT	GGTGAAGAAG	AACATGAAAC	AGAGGAGAAA	ACTGCTACTT	ACACTTTTGC	CAATGCTCCT
18761	GTAAAAGCCG	AGGCTCAAAT	TACAAAAGAG	GGCTTACCAA	TAGGTTTGGA	GATTTTCAGCT	GAAAACGAAT
18831	CTAAACCCAT	CTATGCAGAT	AAACTTTATC	AGCCAGAACCC	TCAAGTGGGA	GATGAAACTT	GGACTGACCT
18901	AGACGGAAAA	ACCGAAGAGT	ATGGAGGCGT	GGCTCTAAAG	CCTACTACTA	ACATGAAACC	CTGTTACGGG
18971	TCCTATGCGA	AGCCTACTAA	TTTAAAAGGT	GGTCAGGCCA	AACCGAAAAA	CTCGGAACCG	TCGAGTGAAA
19041	AAATTGAATA	TGATATTGAC	ATGGAATTTT	TTGATAACTC	ATCGCAAAGA	ACAAACTTCA	GTCTTAAAT
19111	TGTCATGTAT	GCAGAAAAATG	TAGGTTTGGA	AACGCCAGAC	ACTCATGTAG	TGTACAAACC	TGGAACAGAA
19181	GACACAAGTT	CCGAAGCTAA	TTTGGGACAA	CAGTCTATGC	CCAACAGACC	CAACTACATT	GGCTTCAGAG
19251	ATAACTTTAT	TGGACTCATG	TACTATAACA	GTACTGGTAA	CATGGGGGTG	CTGGCTGGCT	AAGCTCTCTA
19321	GTAAATGCA	GTGGTTGACT	TGCAGGACAG	AAACACAGAA	CTTTCTTACC	AACTCTTGCT	TGACTCTCTG
19391	GGCGACAGAA	CCAGATACTT	TAGCATGTGG	AATCAGGCTG	TGGACAGTTA	TGATCCTGAT	GTACGTGTTA
19461	TTGAAAATCA	TGGTGTGGAA	GATGAACCTT	CCAACATTG	TTTTCCACTG	GACGGCATAG	GTGTTCCAAC
19531	AACCAAGTTAC	AAATCAATAG	TTCCAAATGG	AAGAAGATAAT	AATAATTGGA	AAGAACCCTG	AGTAAATGGA
19601	ACAAGTGAGA	TCGGACAGGG	TAATTTGTTT	GCCATGGAAG	TTAACCTTCA	AGCCAATCTA	TGGCGAAGTT
19671	TCCTTTATTC	CAATGTGGCT	CTGTATCTCC	CAGACTCGTA	CAAATACACC	CCGTCCAATG	TCACTCTTCC
19741	AGAAAACAAA	AACACCTACG	ACTACATGAA	CGGGCGGGTG	GTGCGGCCAT	CTCTAGTAGA	CACCTATGTG
19811	AACATTGGTG	CCAGGTGGTC	TCTGGATGCC	ATGGACAATG	TCAACCCATT	CAACCCACCT	CAACCATGCTG
19881	GCTTGCCTTA	CCGATCTATG	CTTCTGGGTA	ACGGACGTTA	TGTGCCTTTC	CACATACAAG	TGCCTCAAAA
19951	ATTCTTTCGT	GTAAAAAAC	TGCTGCTTCT	CCCAGGCTCC	TACACTTATG	AGTGGAACTT	TAGGAAGGAT
20021	GTGAACATGG	TTCTACAGAG	TTCCCTCGGT	AACGACCTGC	GGGTAGATGG	CGCCAGCATC	AGTTTCACGA
20091	GCATCAACCT	CTATGCTACT	TTTTCTCCCA	TGGCTACAAA	CACCGCTTCC	ACCCTTGAAG	CCATCTGCG
20161	GAATGACACC	AATGATCAGT	CATTCAACGA	CTACCTATCT	GCAGCTAACA	TGCTCTACCC	CATTCTGCCC
20231	AATGCAACCA	ATATTCCCAT	TTCCATTCTT	TCTCGCAACT	GGGCGGCTTT	CAGAGGCTGG	TCATTTACCA
20301	GACTGAAAC	CAAAGAAACT	CCCTCTTTGG	GGTCTGGATT	TGACCCCTAC	TTTGTCTATT	CTGGTTCTAT
20371	TCCTACCTG	GATGATACCT	TCTACCTGAA	CCACACTTTT	AAGAAGGTTT	CCATCATGTT	TGACTCTTCA
20441	GTGAGCTGGC	CTGGAATGA	CAGGTTACTA	TCTCCTAACG	AATTTGAAAT	AAAGCGCAT	GTGGATGGCG
20511	AAGGCTACAA	CGTAGCCCAA	TGCAACATGA	CCAAAGACTG	GTCTTTGGTA	CAGATGCTCG	CCAACTACAA
20581	CATCGGCTAT	CAGGGCTTCT	ACATTCCAGA	AGGATACAAA	GATCGCATGT	ATTCATTTTT	CAGAAACTTC
20651	CAGCCCATGA	GCAGGCAAGT	GGTTGATGAG	GTCAATTACA	AAGACTTCAA	GGCCGTGCGC	ATACCTTACC
20721	AACACAACAA	CTCTGGCTTT	GTGGGTTACA	TGGCTCCGAC	CATGCGCCAA	GGTCAACCC	ATCCCGCTAA
20791	CTATCCCTAT	CCACTCATTG	GAACAACATG	CGTAAATAGT	GTTACGCAGA	AAAAGTTCTT	GTGTGACAGA
20861	ACCATGTGGC	GCATACCGTT	CTCGAGCAAC	TTTATGCTTA	TGGGGGCCCT	TACAGACTTG	GGACAGAATA
20931	TGCTCTATGC	CAACTCAGCT	CATGCTCTGG	ACATGACCTT	TGAGGTGGAT	CCCATGGATG	AGCCCACCCT
21001	GCTTTATCTT	CTCTTCGAAG	TTTTCGACGT	GGTCAGAGTG	CATCAGCCAC	ACCGCGGCAT	CATCGAGGCA
21071	GTCTACCTGC	GTACACCGTT	CTCGGCCGGT	AACGCTACCA	CGTAAGAAGC	TTCTTGCTTC	TTGCAATAG
21141	CAGCTGCAAC	CATGGCCCTG	GGATCCCAAA	ACGGCTCCAG	CGAGCAAGAG	CTCAGAGCCA	TTGTCCAAGA
21211	CCTGGGTTGC	GGACCCCTAT	TTTTGGGAAC	CTACGATAAG	CGCTTCCCGG	GGTTTATGGC	CCCCGATAAG
21281	CTCGCCTGTG	CCATTGTAAA	TACGGCCGGA	CGTGAGACGG	GGGGAGAGCA	CTGGTTGGCT	TTGCGTTGGA
21351	ACCCACGTTT	TAACACCTGC	TACCTTTTTG	ATCCTTTTGG	ATTCTCGGAT	GATCGTCTCA	AACAGATTTA
21421	CCAGTTTGAA	TATGAGGGTC	TCCTGCCTCG	CAGCGCTCTT	GCTACCAAGG	ACCGCTGTAT	TACGCTGGAA
21491	AAATCTACCC	AGACCGTGCA	GGGCCCCCGT	TCTGCCGCTT	GCGGACTTTT	CTGCTGCATG	TTCTTTCACG
21561	CCTTTGTGCA	CTGGCCTGAC	CGTCCCATGG	ACGGAACCC	CACCATGAAA	TTGCTAAGT	GAGTGCCAAA
21631	CAACATGCTT	CATTCTCCTA	AAGTCCAGCC	CACCCGTGTG	GACAATCAAA	AAGCACTCTA	CCATTTTCTT
21701	AATACCCATT	CGCCTTATTT	TCGCTCTCAT	CGTACACACA	TCGAAAGGGC	CACTGCGTTC	GACCGTATGG
21771	ATGTTCAATA	ATGACTCATG	TAAACAACGT	GTTCAATAAA	CATCACTTTA	TTTTTTTACA	TGTATCAAGG
21841	CTCTGGATTA	CTTATTATT	TACAAGTCGA	ATGGGTTCTG	ACGAGAATCA	GAATGACCCG	CAGGAGTGGA
21911	TACGTTGCGG	AACTGATATT	TGGGTTGCCA	CTTGAATTCG	GGAATCACCA	ACTTGGGAAC	CGGTATATCG
21981	GGCAGGATGT	CACTCCACAG	CTTTCTGGTC	AGCTGCAAG	CTCCAAGCAG	GTCAGGAGCC	GAAATCTTGA
22051	AATCACAAAT	AGGACCAAGT	CTCTGAGCGC	GAGAGTTGCG	GTACACCGGA	TTGCAGCACT	GAAACACCAT
22121	CAGCGACGGA	TGTCTCACGC	TTGCCAGCAC	GGTGGGATCT	GCAATCATGC	CCACATCCAG	ATCTTCAGCA
22191	TTGGCAATGC	TGAACGGGGT	CATCTTGCA	GTCTGCCTAC	CCATGGCGGG	CACCCAATTA	GGCTTGTTGGT

Figure 6, contd.

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22401 CTGCTCGAAA ACTGGTTAGC TGCACAGCCG GCATCATTCA CACAGCAGCG GCGCTCATTG TTGGCTATTT
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23871 AAGTGCCCAT CAGTGTGGAA GAGCTCAGCT GCGCTACGA GCTTAACTTT TTTTCACTC GTACTCCCC
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26601 CCCGATGAA TTGGCTCAGC GCCGGGCTT CTATGATTTT TCGAGTTAAT GATATACGCG CCTACCGAAA
26671 CCAAATACTT TTGGAACAGT CAGCTCTTAC CACCACGCCC CGCCAACACC TTAATCCAG AAATTGGCCC

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Figure 6, contd.

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Figure 6, contd.

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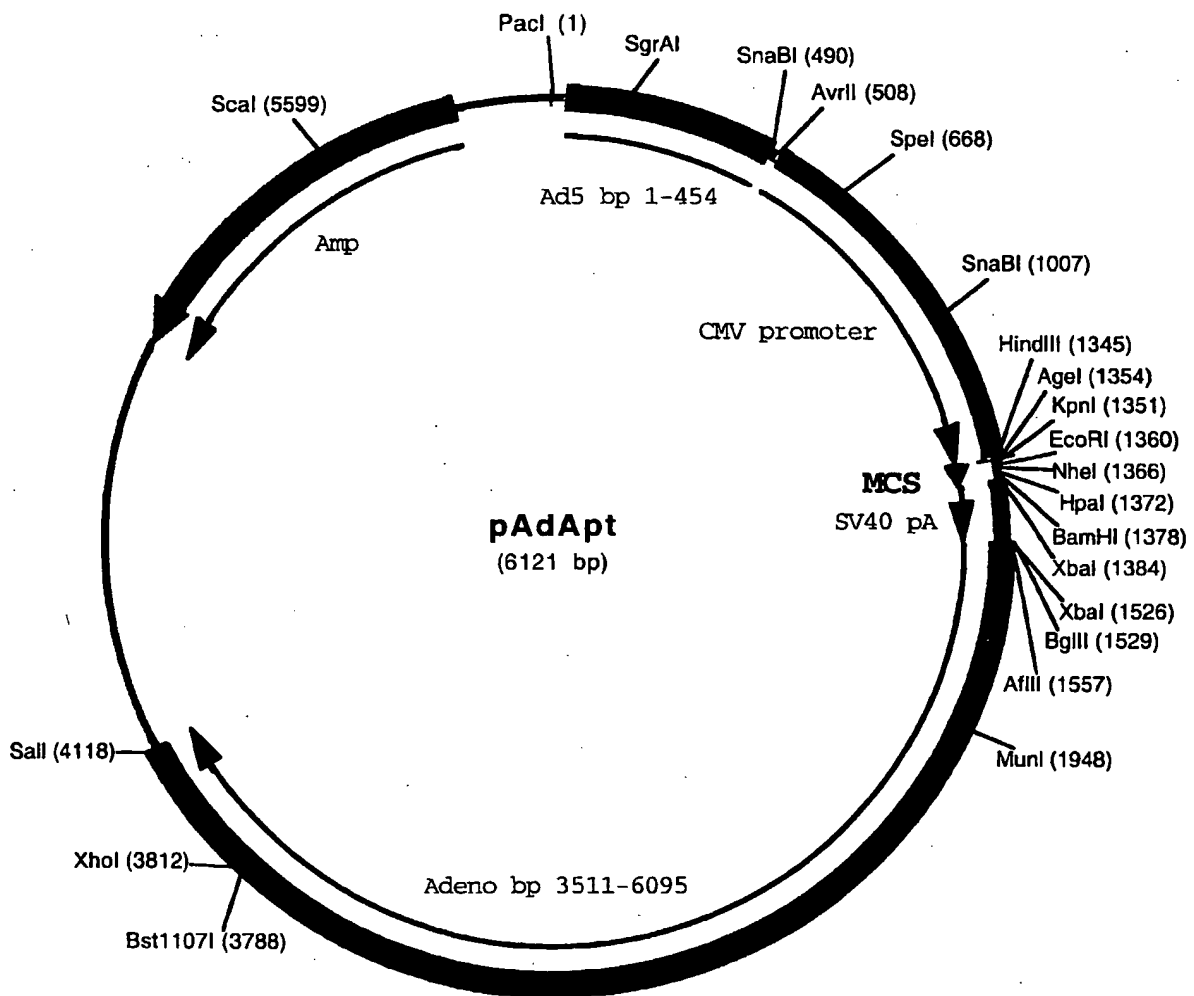


Figure 7

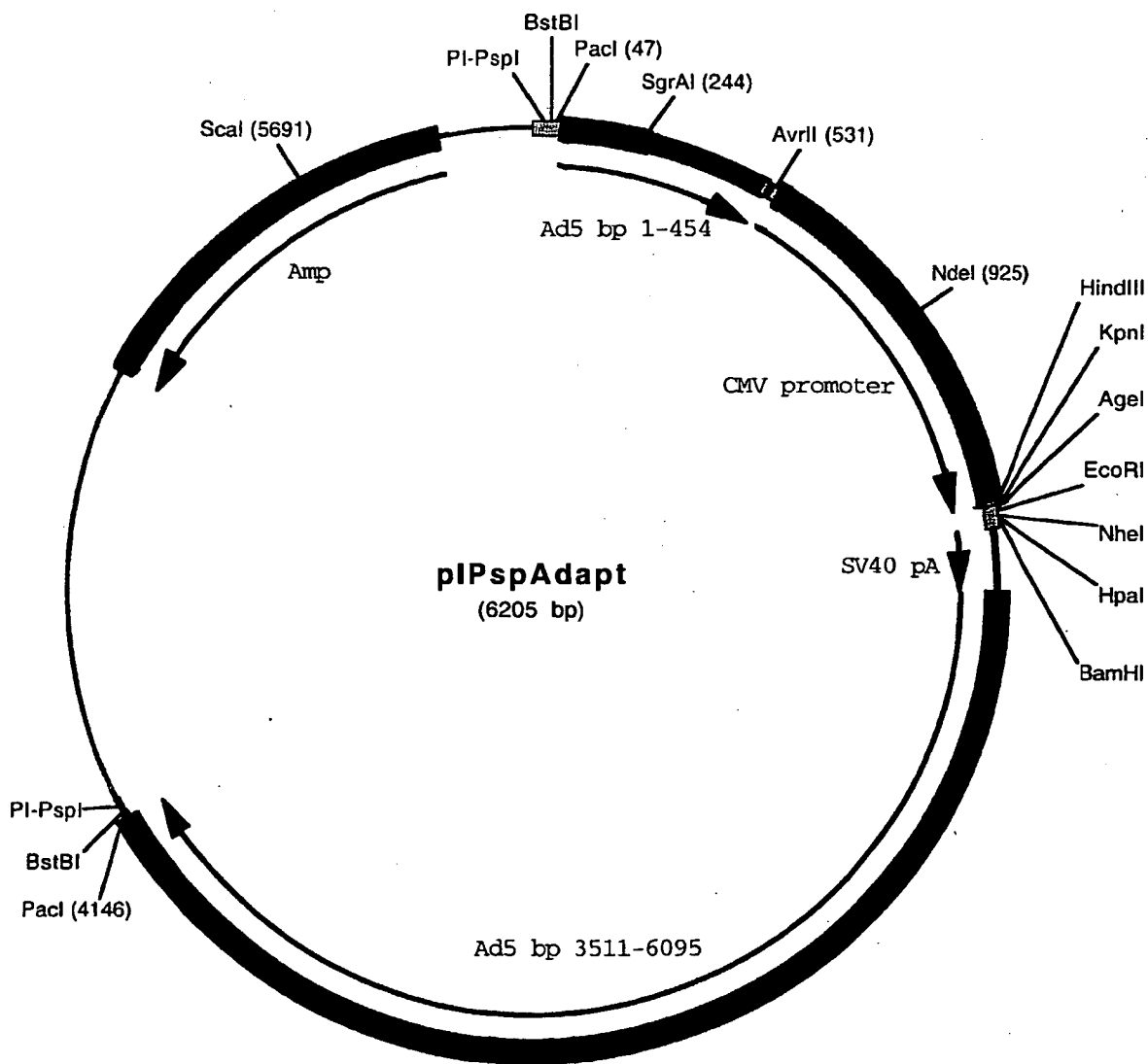


Figure 8



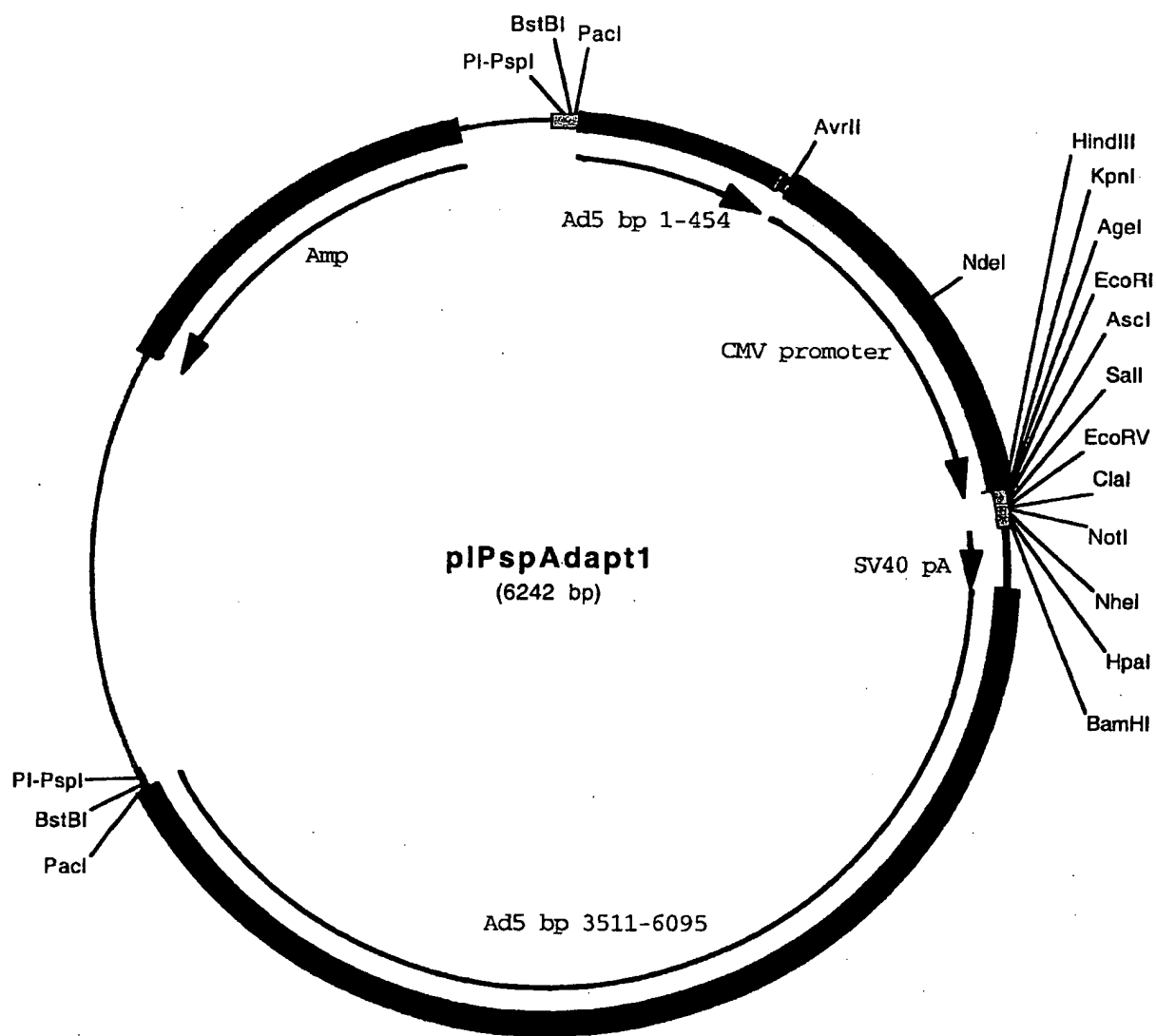


Figure 9

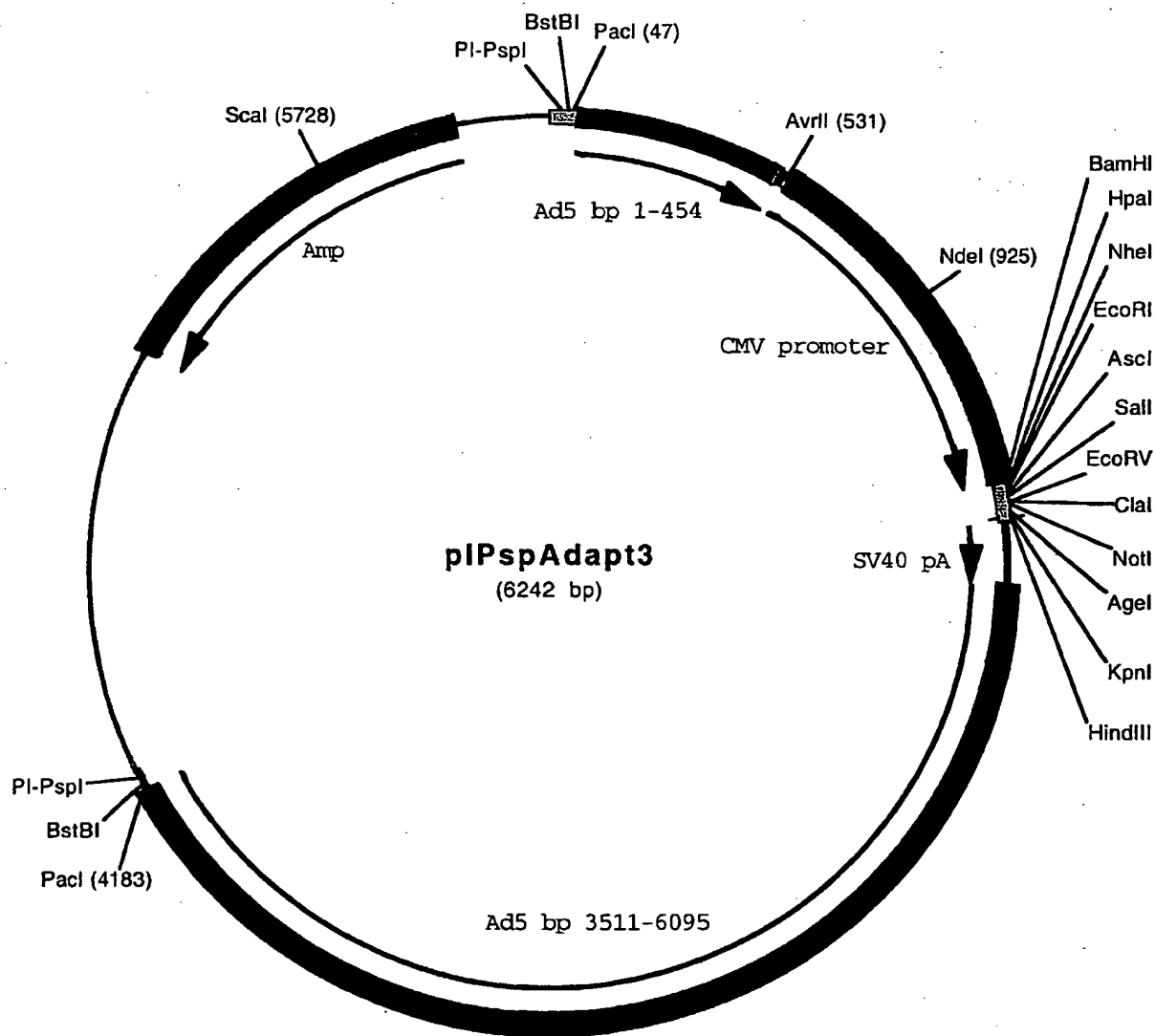


Figure 10

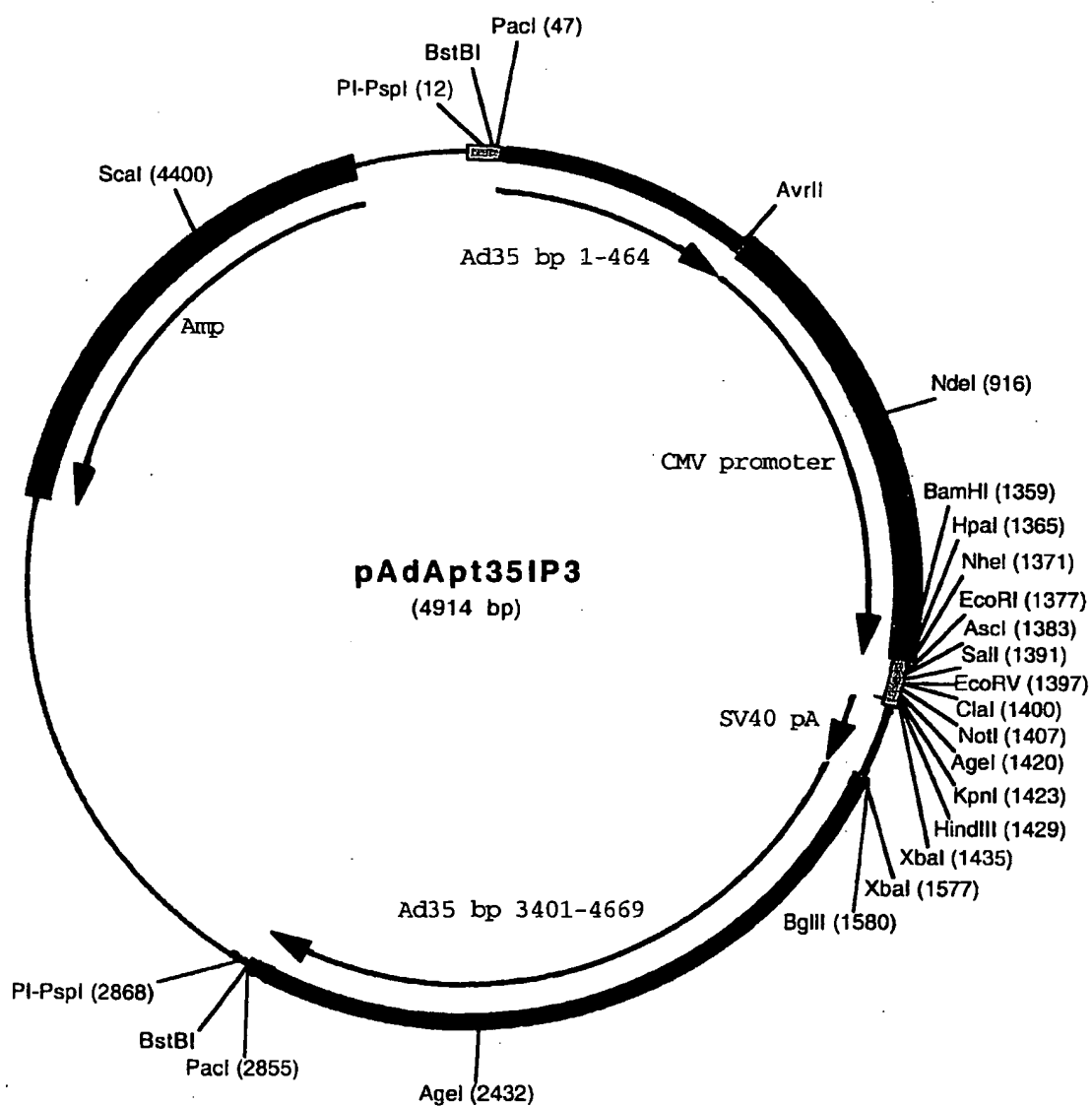


Figure 11

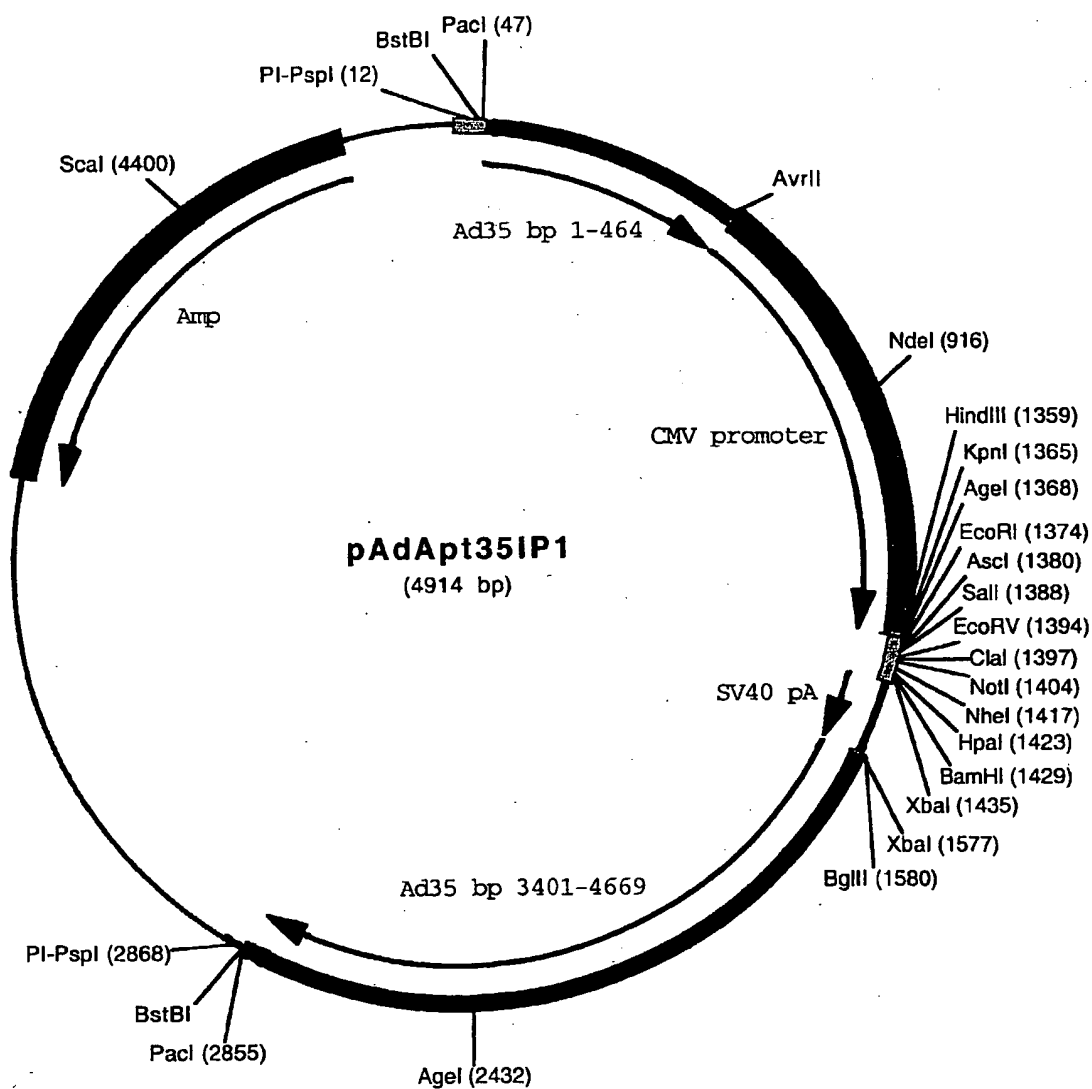


Figure 12

# Construction of cosmid vector pWE.Ad35.pIX-rITR

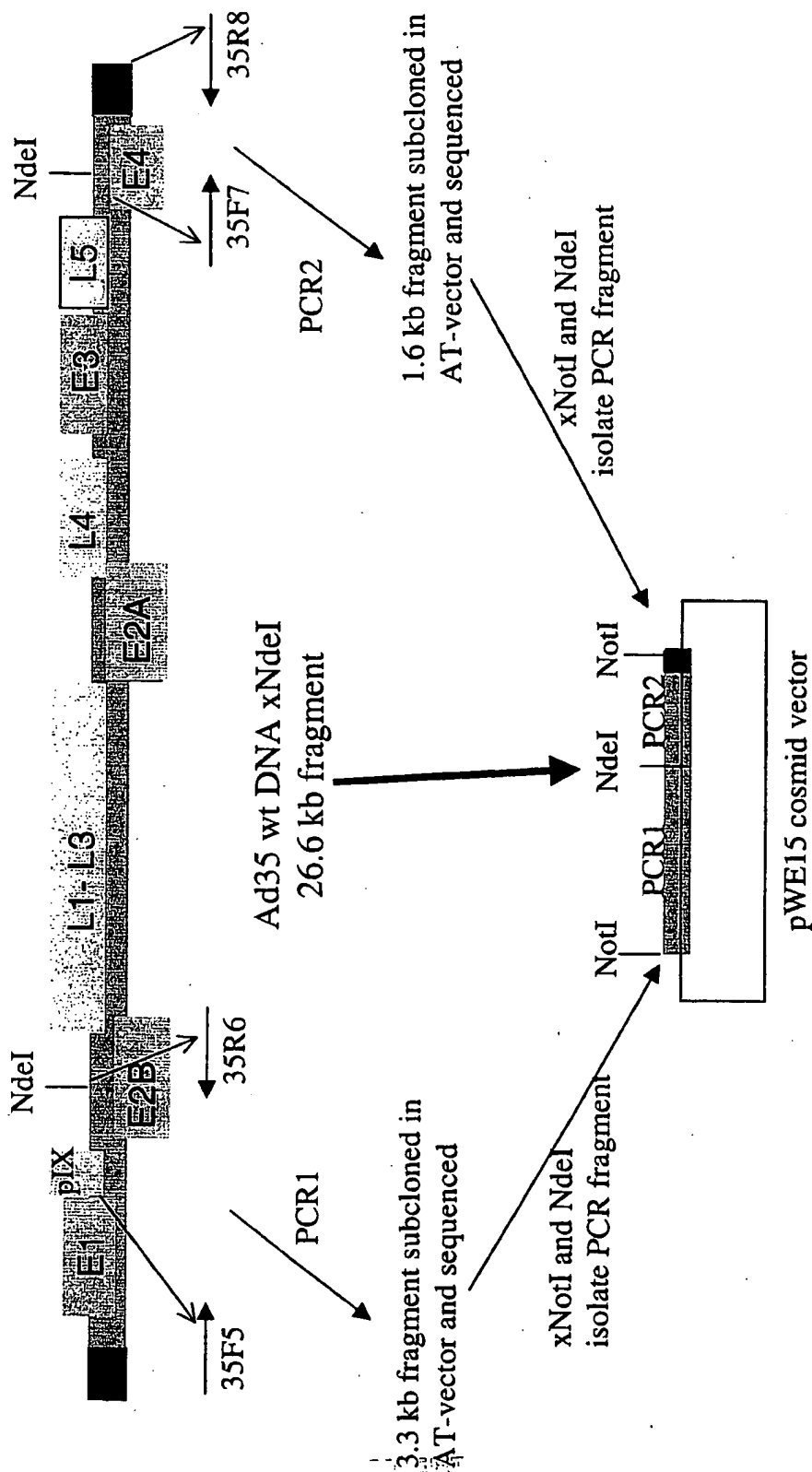


Figure 13

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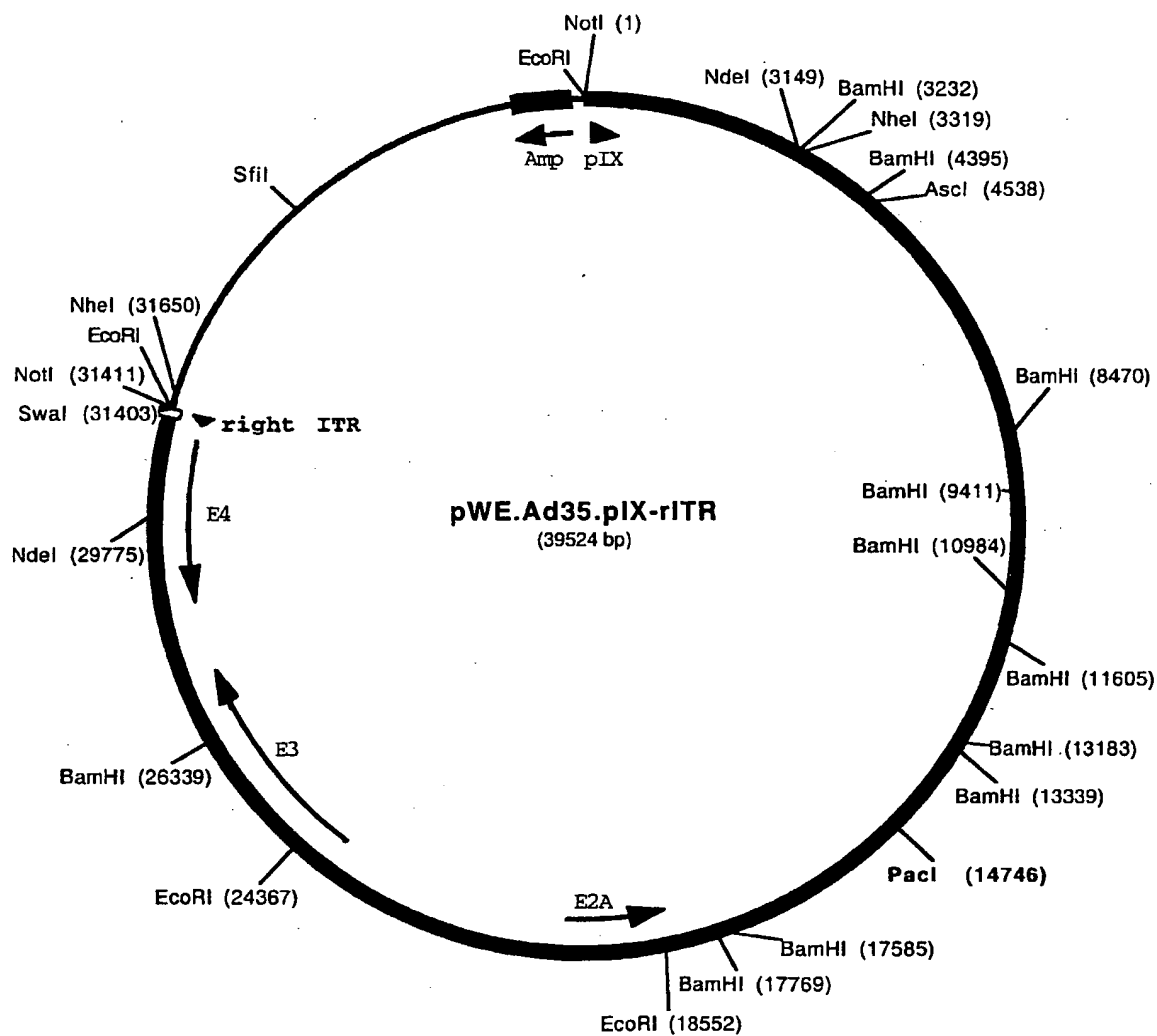


Figure 14

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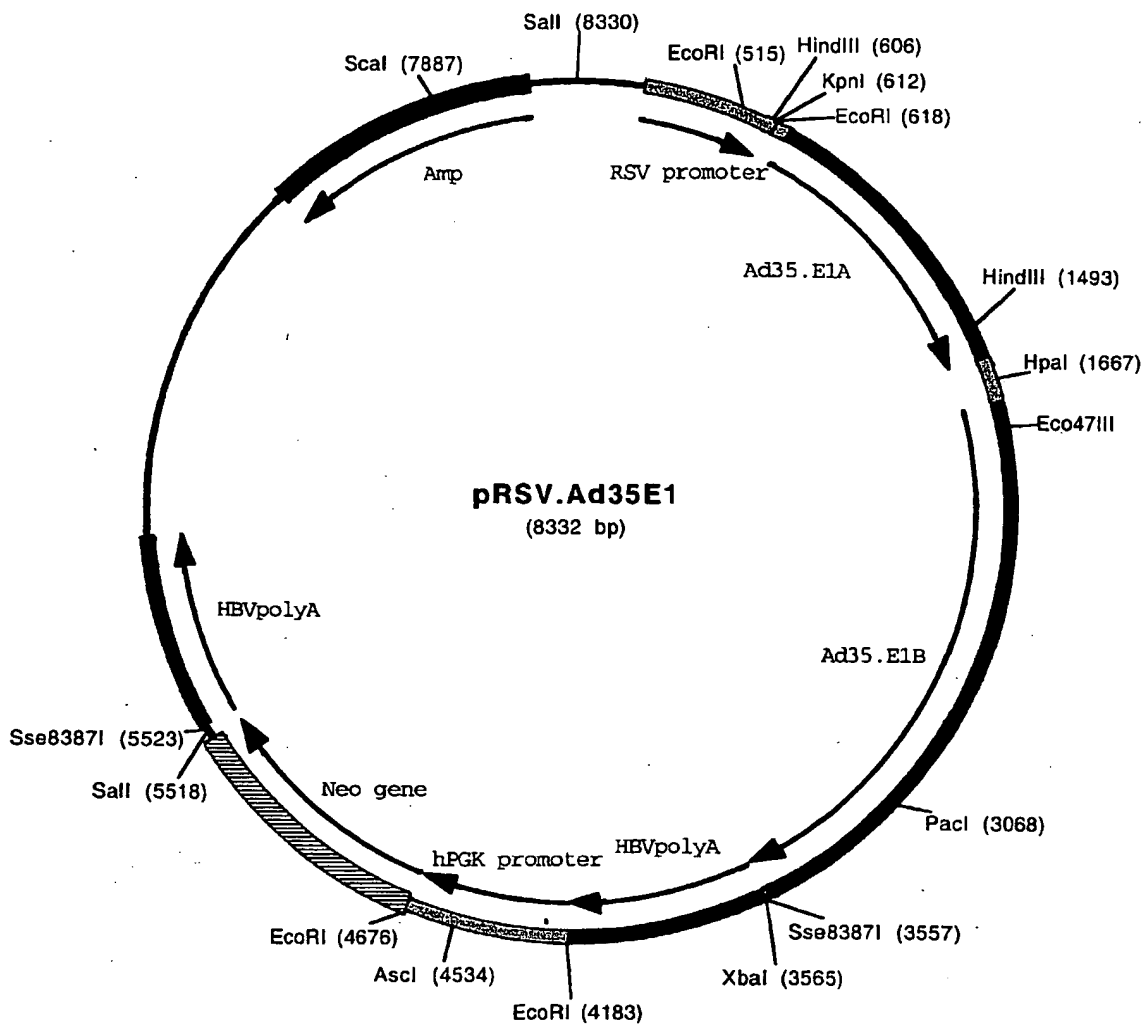


Figure 15

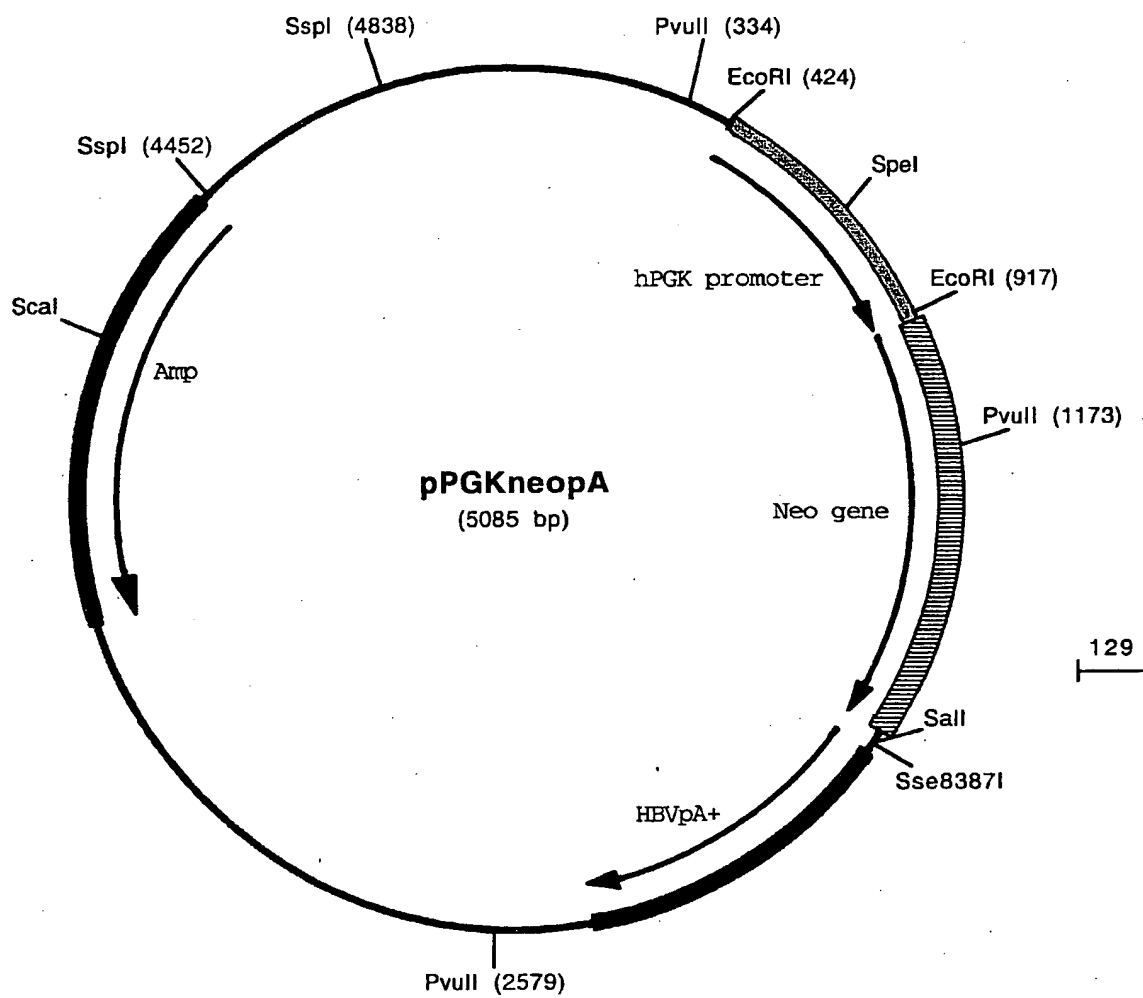


Figure 16



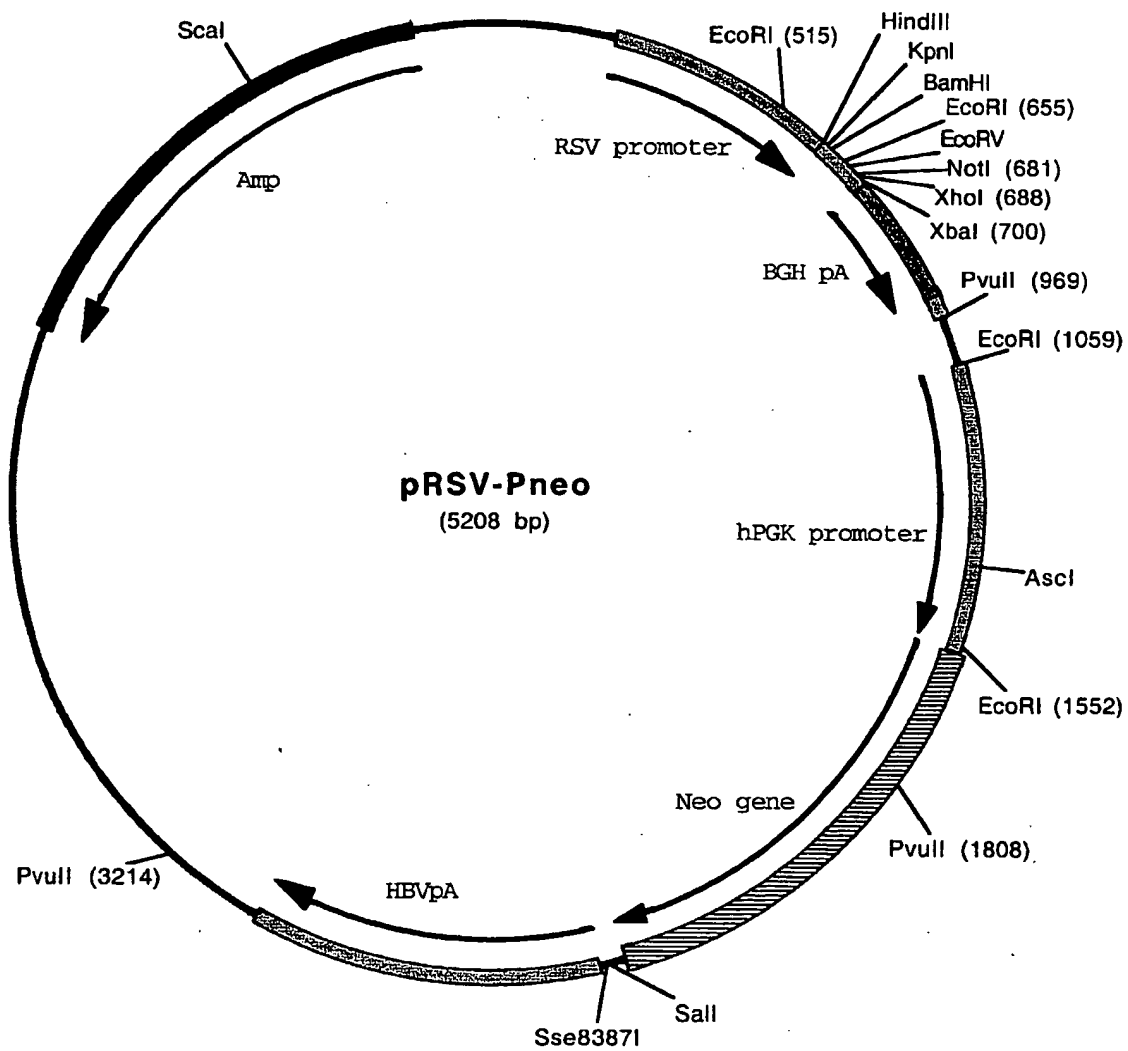


Figure 17

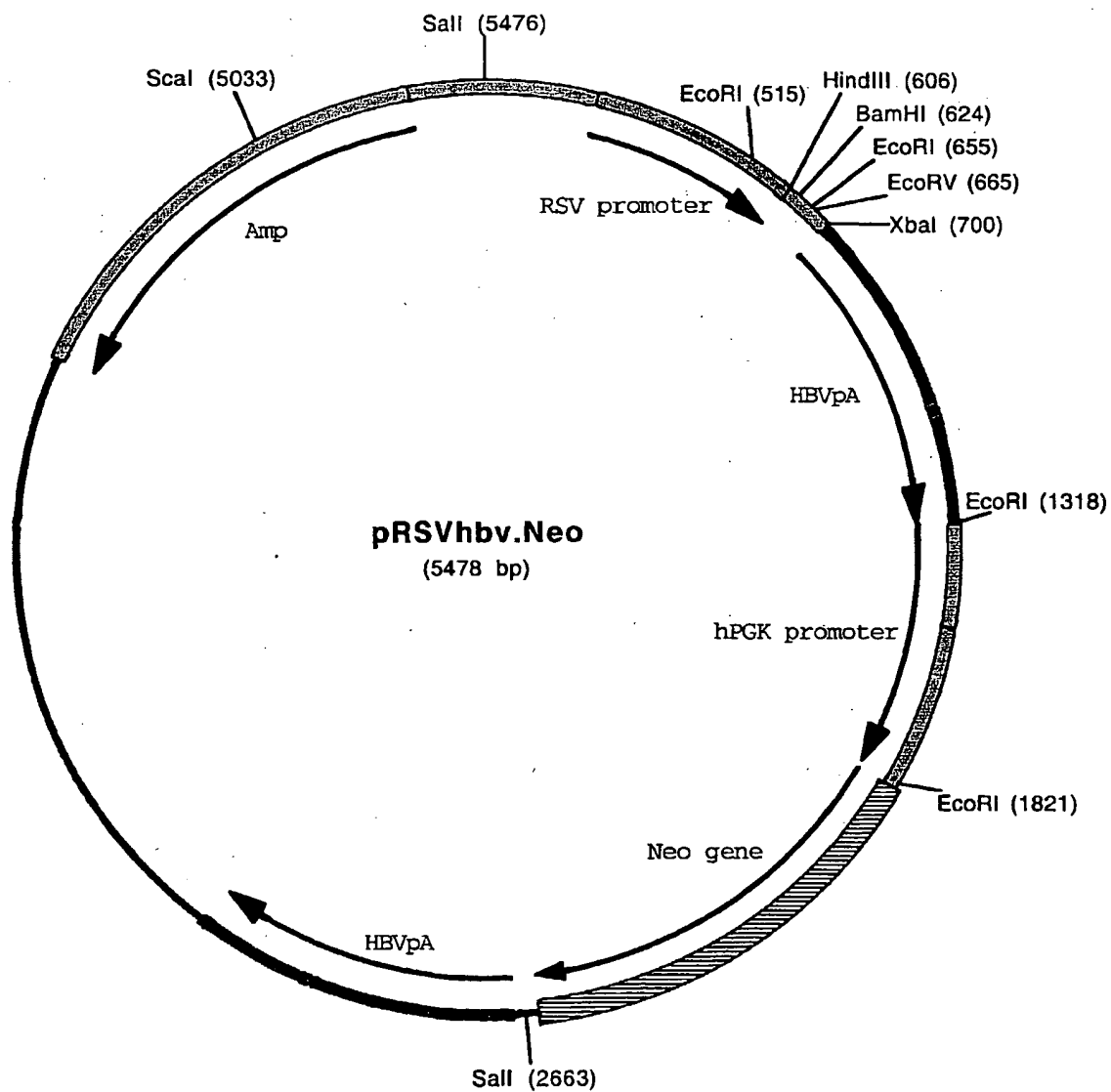
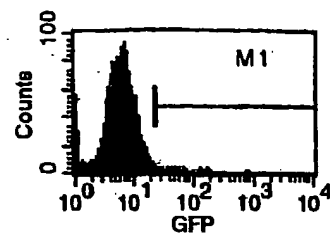


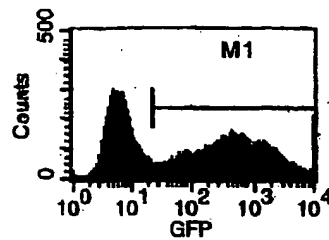
Figure 18

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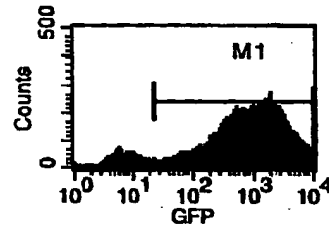
Figure 19



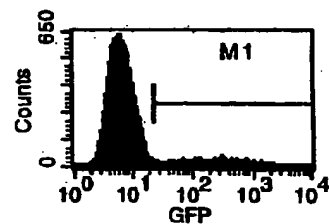
control



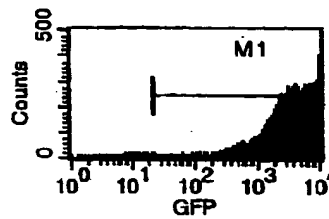
Ad 5



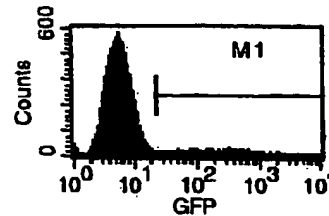
Ad5fib16



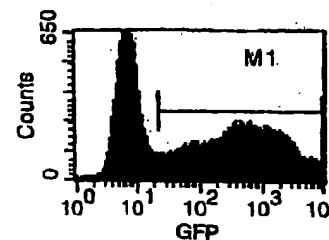
Ad5fib17



Ad5fib35



Ad5fib40-L



Ad5fib51

Figure 20

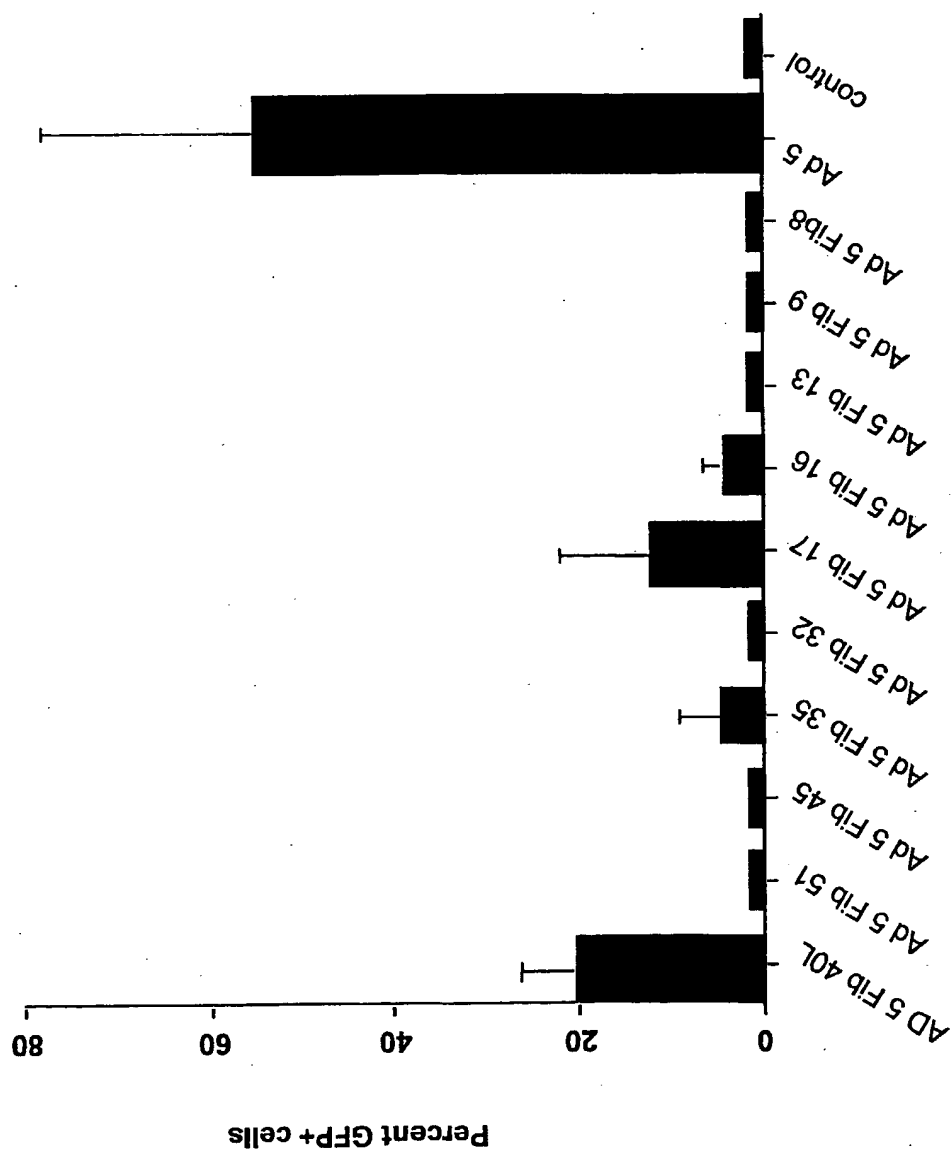
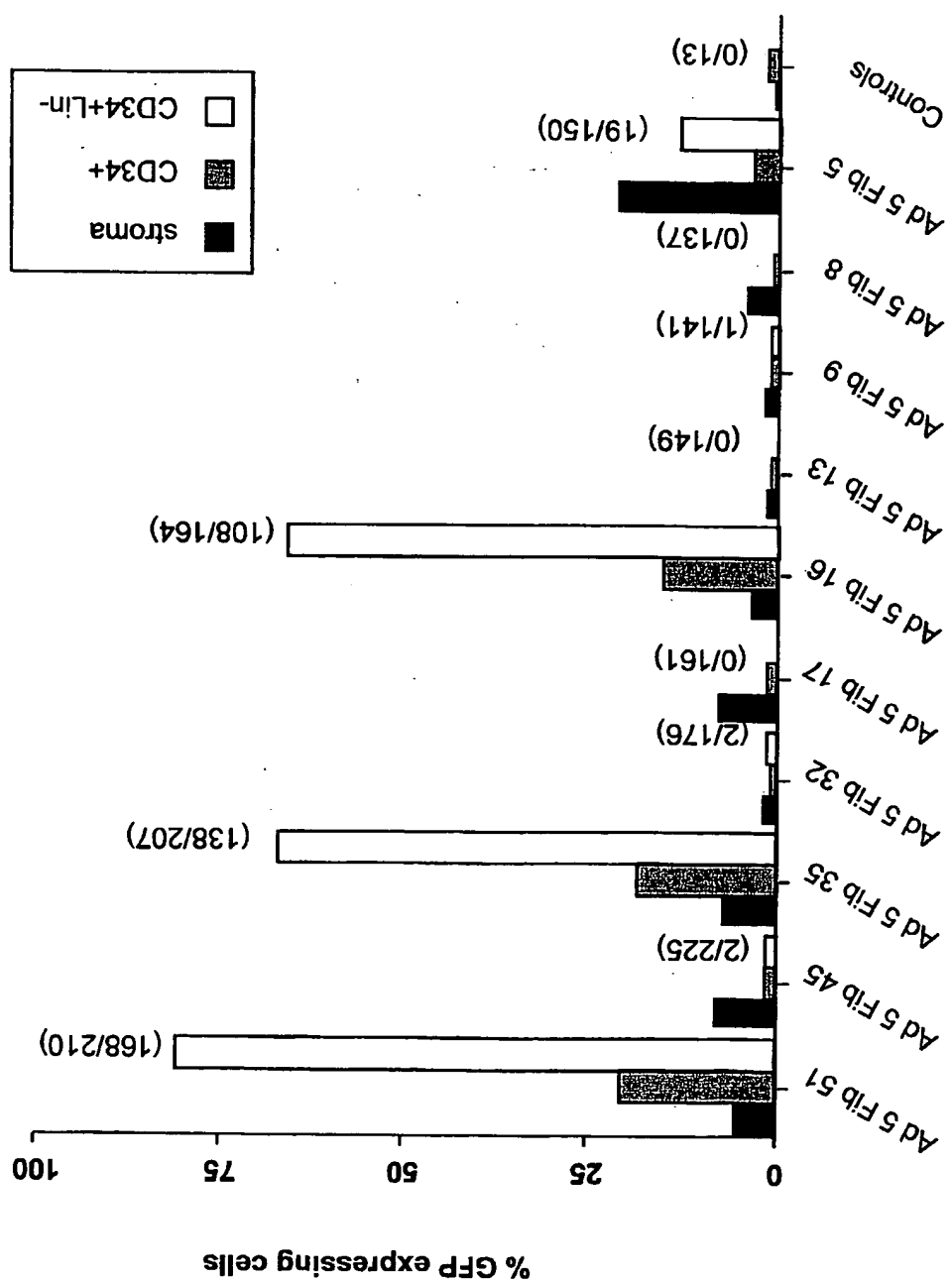


Figure 21



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Figure 22a

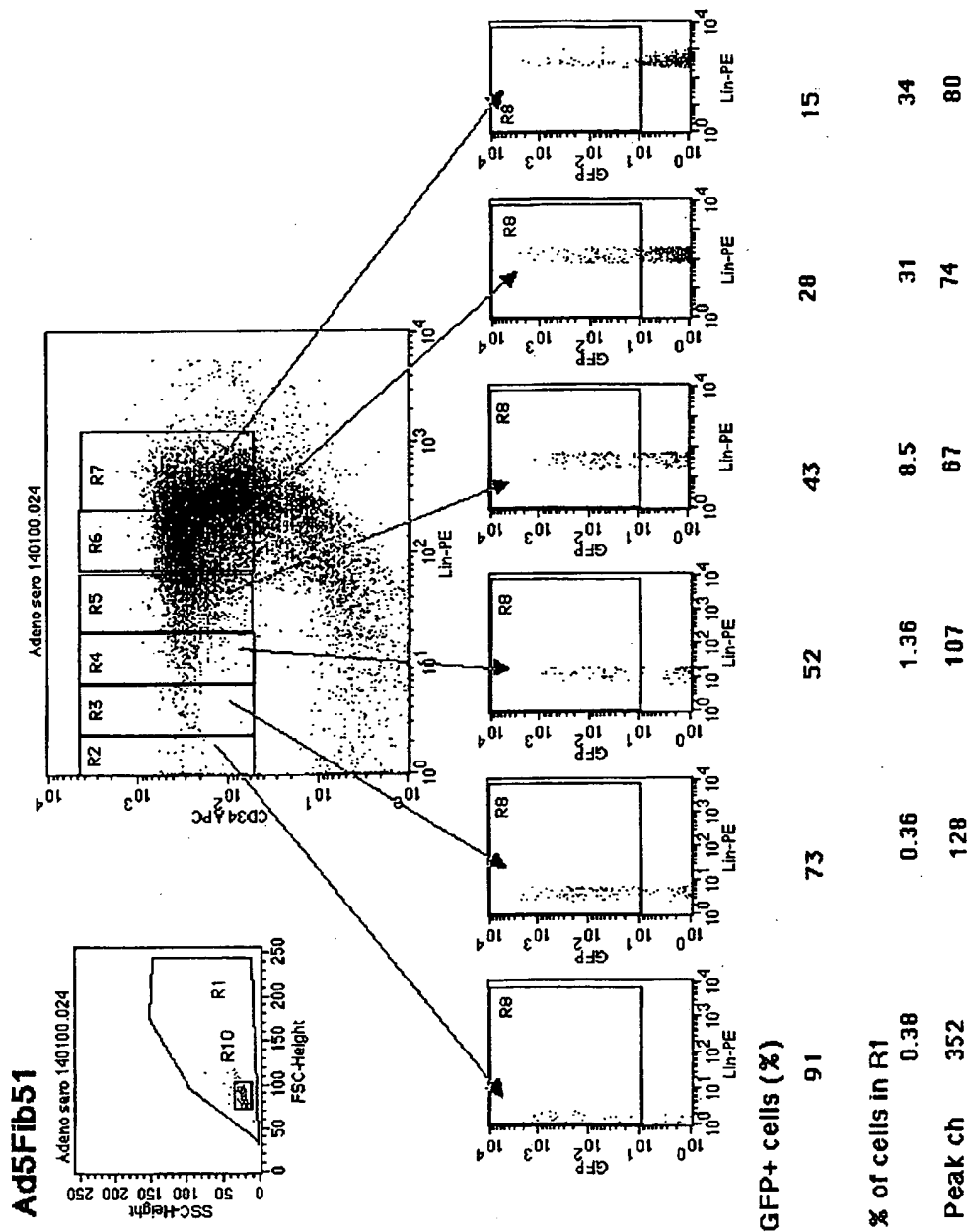


Figure 22b

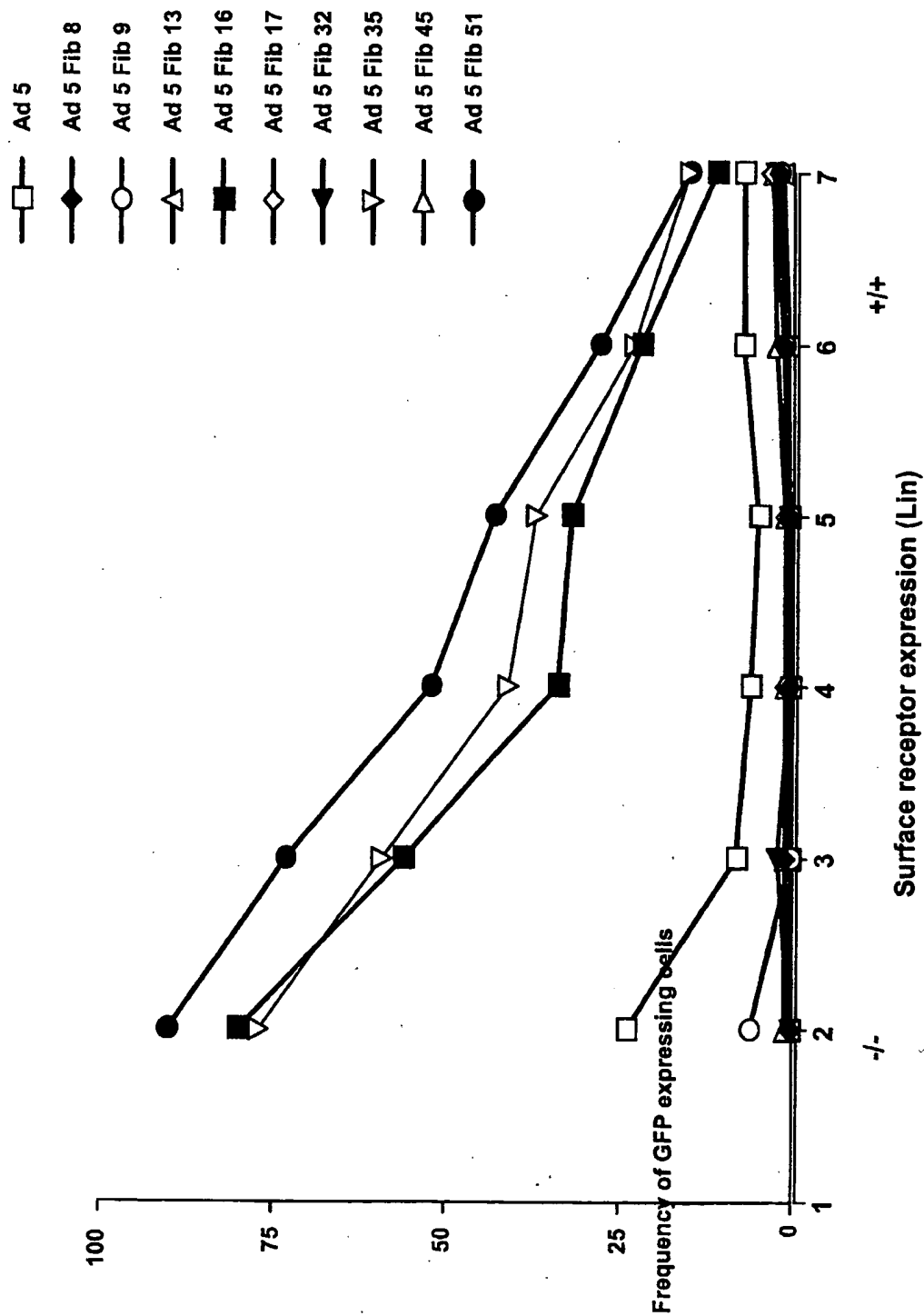


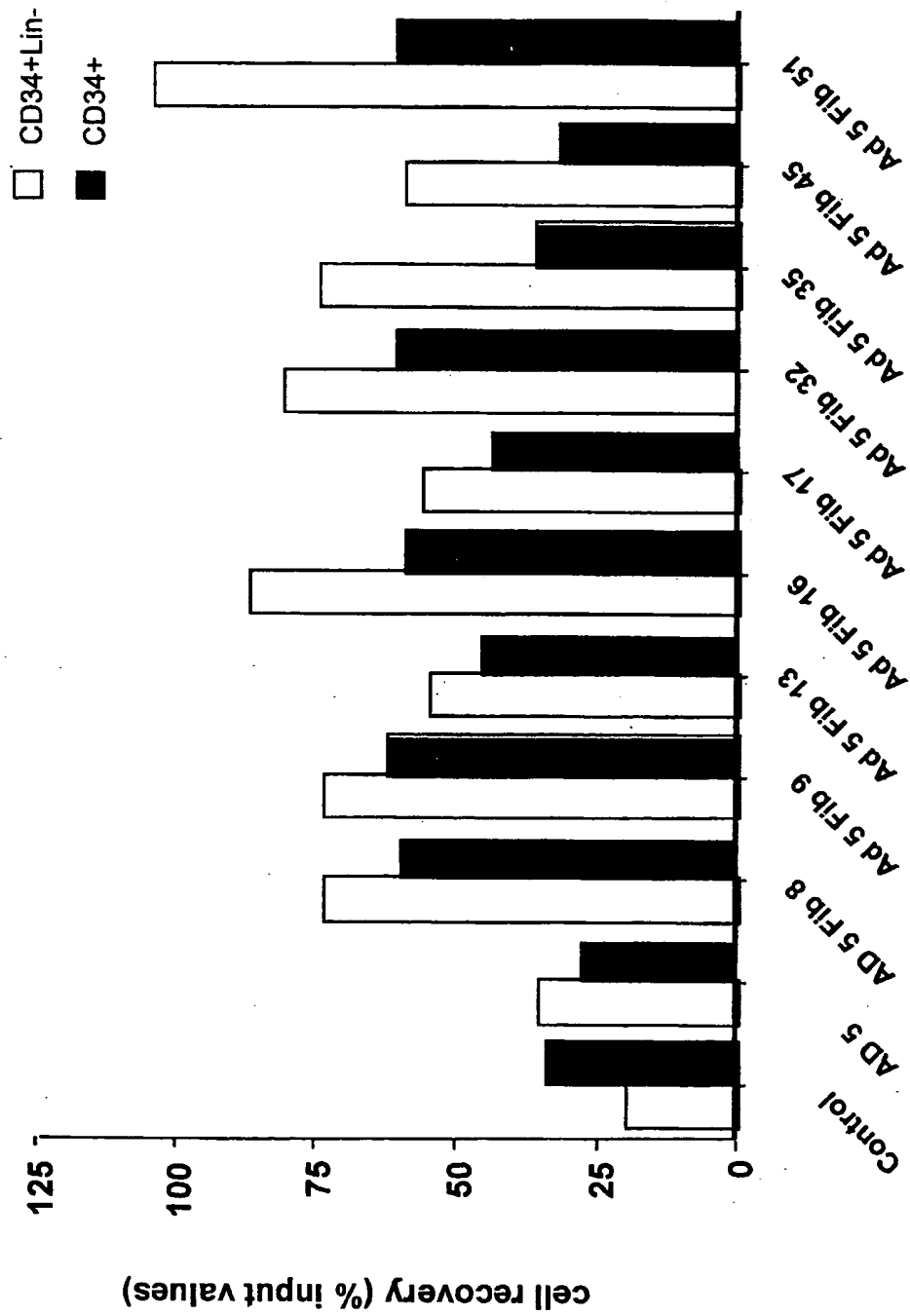
FIGURE 23

1	MLLQMKRRARPS	EDTFNPVYPY	DTETGPPTV	PFLT	PPFV	SP	Fib 5 protein						
1	MLLQMKRRARPS	EDTFNPVYPY	EDSSSQH	PFIN	PGFI	SS	Fib 16 protein						
1	MLLQMKRRARPS	EDTFNPVYPY	EDSSSQH	PFIN	PGFI	SS	Fib 35 protein						
1	MLLQMKRRARPS	EDTFNPVYPY	EDSSSQH	PFIN	PGFI	SS	Fib 51 protein						
41	NGFOES	PPGVLS	LRLESE	PLVTS	NGMLA	LKMG	NGLSLDEAG	Fib 5 protein					
40	NGFAQSP	PGVLT	LKCVNP	LT	TASG	PLQ	LKVGS	SLTVDTID	Fib 16 protein				
40	NGFTQSP	PGVLT	LKCLTP	LT	TTGGS	LQ	LKVGG	GGLTVDDTD	Fib 35 protein				
40	NGFTQSP	PGVLT	LKCLTP	LT	TTGGS	LQ	LKVGG	GGLTVDDTD	Fib 51 protein				
81	NLTSON	VTTVSP	PLKKT	KSIN	LEIS	APLTV	TSEALT	VAA	Fib 5 protein				
80	GSLEBN	IT	AAAP	LT	KT	NHSL	IGLL	IGSG	LQT	Fib 16 protein			
80	GTLOBN	IR	ATAPI	TK	NNH	SVELS	IGN	GLET		Fib 35 protein			
80	GTLOBN	IR	ATAPI	TK	NNH	SVELS	IGN	GLET		Fib 51 protein			
121	AAFLMV	AGNTLT	MQSQ	APLT	VHDS	KL	SIAT	OGPL	TVSE	GK	Fib 5 protein		
110											Fib 16 protein		
110											Fib 35 protein		
110											Fib 51 protein		
161	LALQT	SGPL	TTTT	DSST	LTIT	ASPL	TTAT	GS	LGID	LKEPI	Fib 5 protein		
129	LQ										Fib 16 protein		
116											Fib 35 protein		
116											Fib 51 protein		
201	YTQNG	KLGL	KYGAP	LHV	TDDL	NLT	LT	VAT	GPGV	TINNTS	LO	Fib 5 protein	
132												Fib 16 protein	
116												Fib 35 protein	
116												Fib 51 protein	
241	TKVTG	ALGF	DSQGN	MLNV	AGGL	RIDS	QNRRL	I	LDV	SY	PF	Fib 5 protein	
132												Fib 16 protein	
116												Fib 35 protein	
116												Fib 51 protein	
281	DAQNL	NLRL	LG	QGPL	FINS	AHNL	DIN	YNK	GLYL	FTAS	NNS	Fib 5 protein	
132												Fib 16 protein	
116												Fib 35 protein	
116												Fib 51 protein	
321	KKLEV	NLST	AKGL	MFDA	TAIA	INAG	DGLE	FGSP	NAPN	TNP		Fib 5 protein	
137												Fib 16 protein	
116												Fib 35 protein	
116												Fib 51 protein	
361	LKTKI	HG	LEFDS	NKAM	VPK	LGT	GLS	FDST	GAIT	VG	NKNN	Fib 5 protein	
137	LITK											Fib 16 protein	
116												Fib 35 protein	
116												Fib 51 protein	
401	DKLT	LW	TPAP	SP	NCRL							Fib 5 protein	
166												Fib 16 protein	
137												Fib 35 protein	
137												Fib 51 protein	
438	ATVSV	LAVK	GS	LAPI								Fib 5 protein	
203	GYIT	LMG	ASEY	TNTL	PKNN	QVTI	DVNL	AFD	NTG	QIT	YLS	Fib 16 protein	
174	GYV	SLV	GV	SDTV	NQM	F	TQKT	ANI	QLRL	YFD	SSGN	LLTDES	Fib 35 protein
174	GYV	SLV	GV	SDTV	NQM	F	TQKT	ANI	QLRL	YFD	SSGN	LLTDES	Fib 51 protein
477	LDPEY	WNFR	NGDL	TEGT	AYTN	AVGF	MPNLS	AYPK	SHG	KTA		Fib 5 protein	
243	SLKSN	LN	FKDN	QNMAT	GTIT	TSAR	GFMP	STTAY	PF			Fib 16 protein	
214	DLKI	PLKN	KSS	STA	TSET	VASS	KAF	MPSTTAY	PF			Fib 35 protein	
214	NLKI	PLKN	KSS	STA	TSET	VASS	KAF	MPSTTAY	PF			Fib 51 protein	
517	KSNI	VSO	VY	LN	GD							Fib 5 protein	
280	ATET	LN	EDY	IY	GECY	YK	ST	NGTL	FP	PLKV	TV	TLNRRMLAS	Fib 16 protein
250	TRDS												Fib 35 protein
250	TRDS												Fib 51 protein
548	DTTP	SAYS	MS	FS	SWD	SGH	NYINE						Fib 5 protein
319	GM												Fib 16 protein
288	NV												Fib 35 protein
288	NV												Fib 51 protein
585	E												Fib 5 protein
356	D												Fib 16 protein
325	D												Fib 35 protein
325	D												Fib 51 protein



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Figure 24



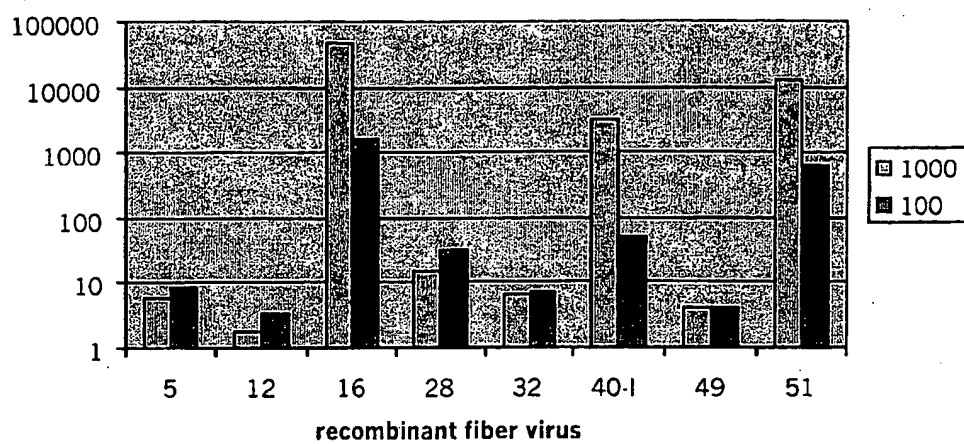
**Figure 25**

Figure 26

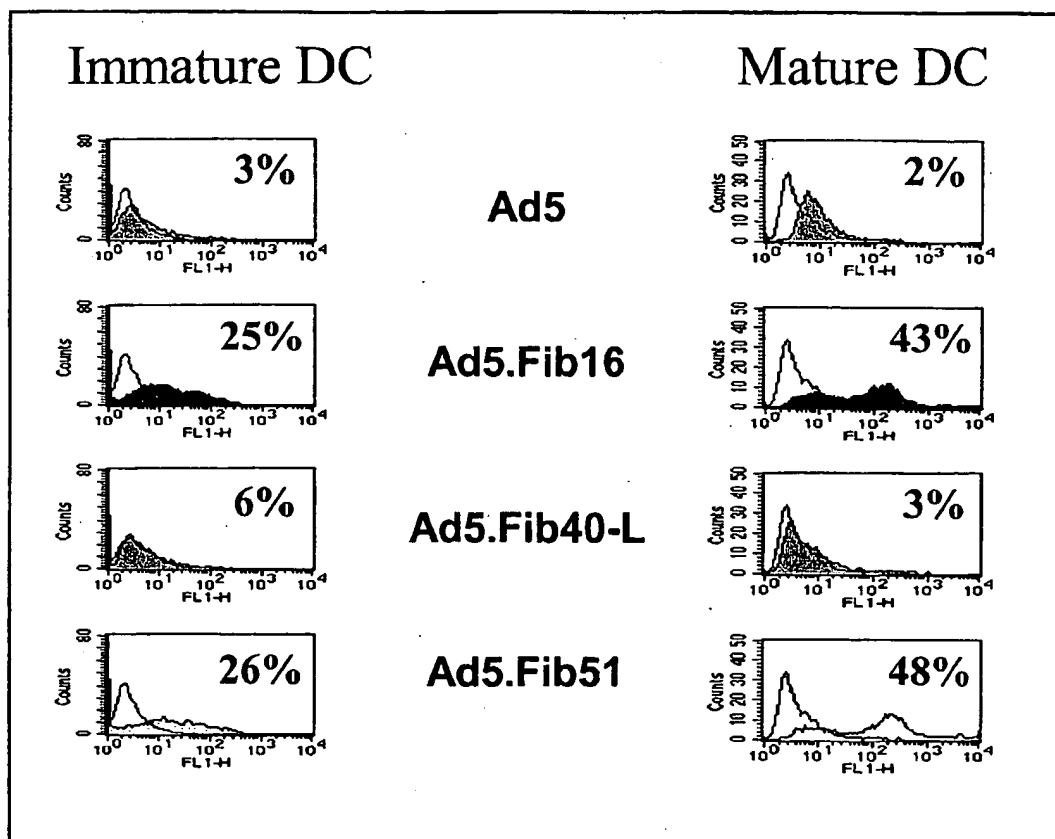


Figure 27

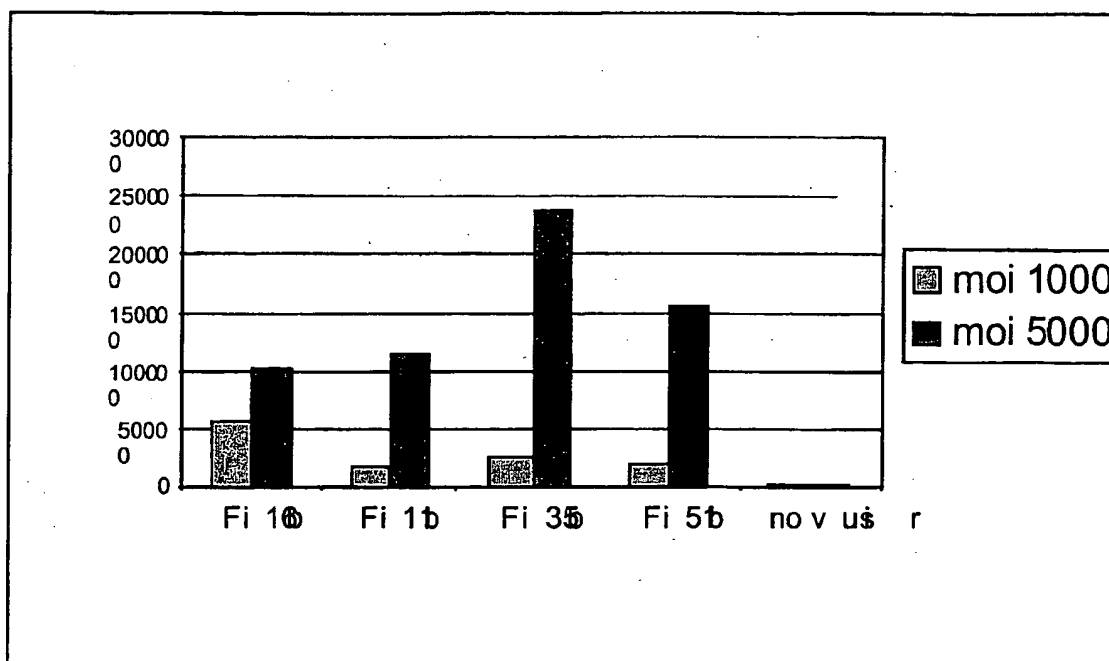


Figure 28

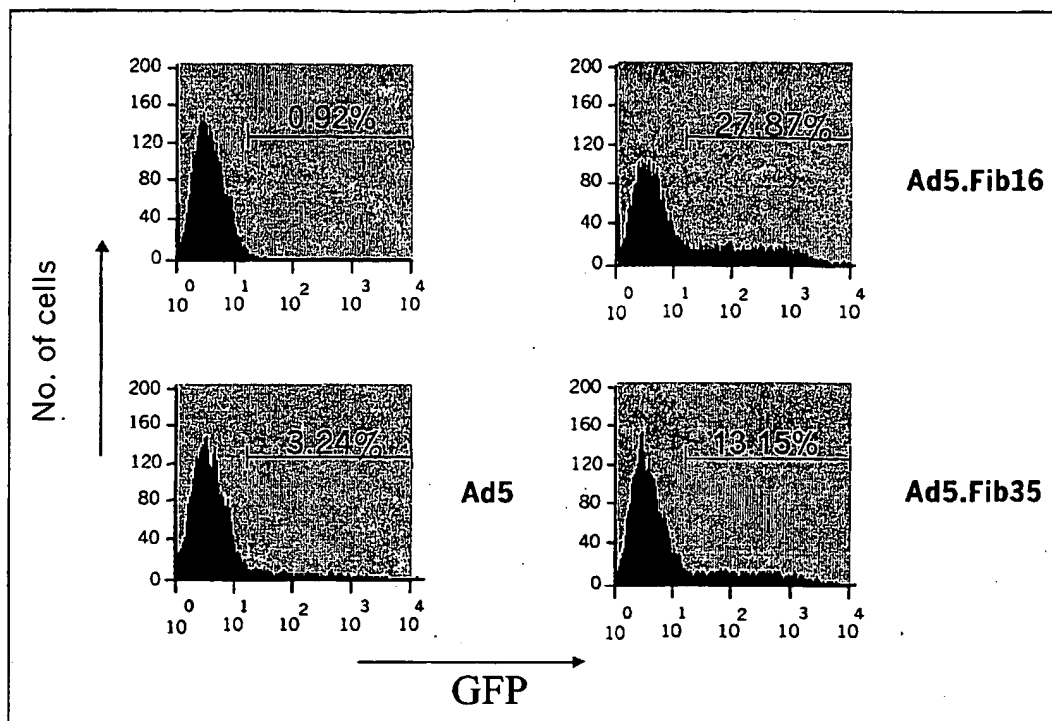
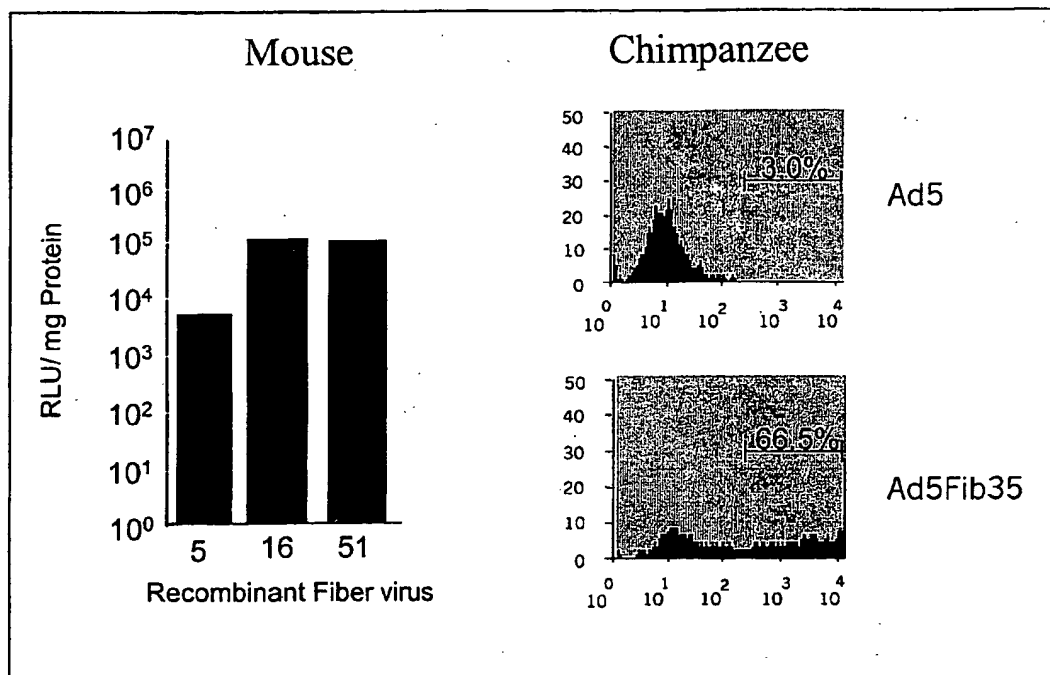
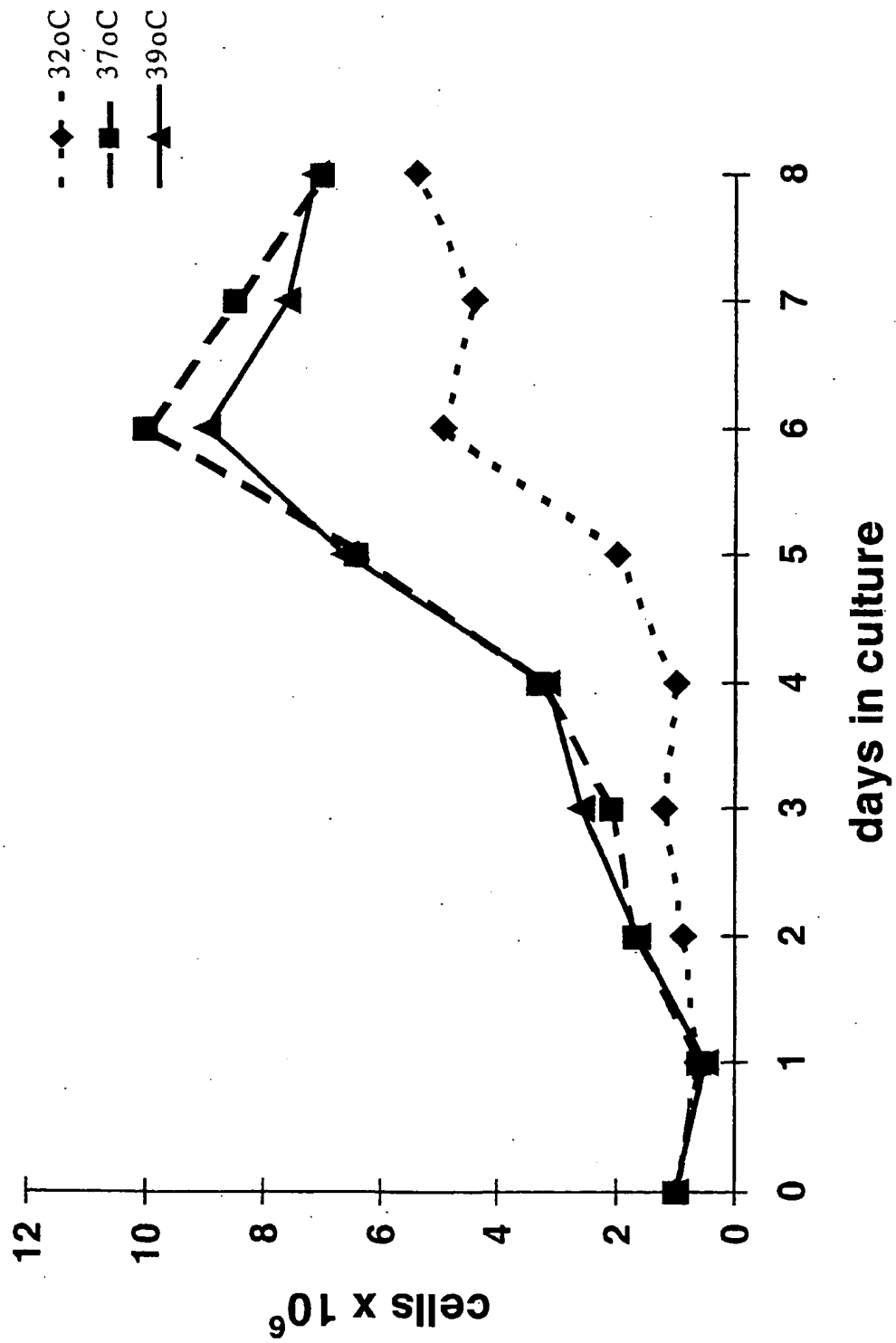


Figure 29



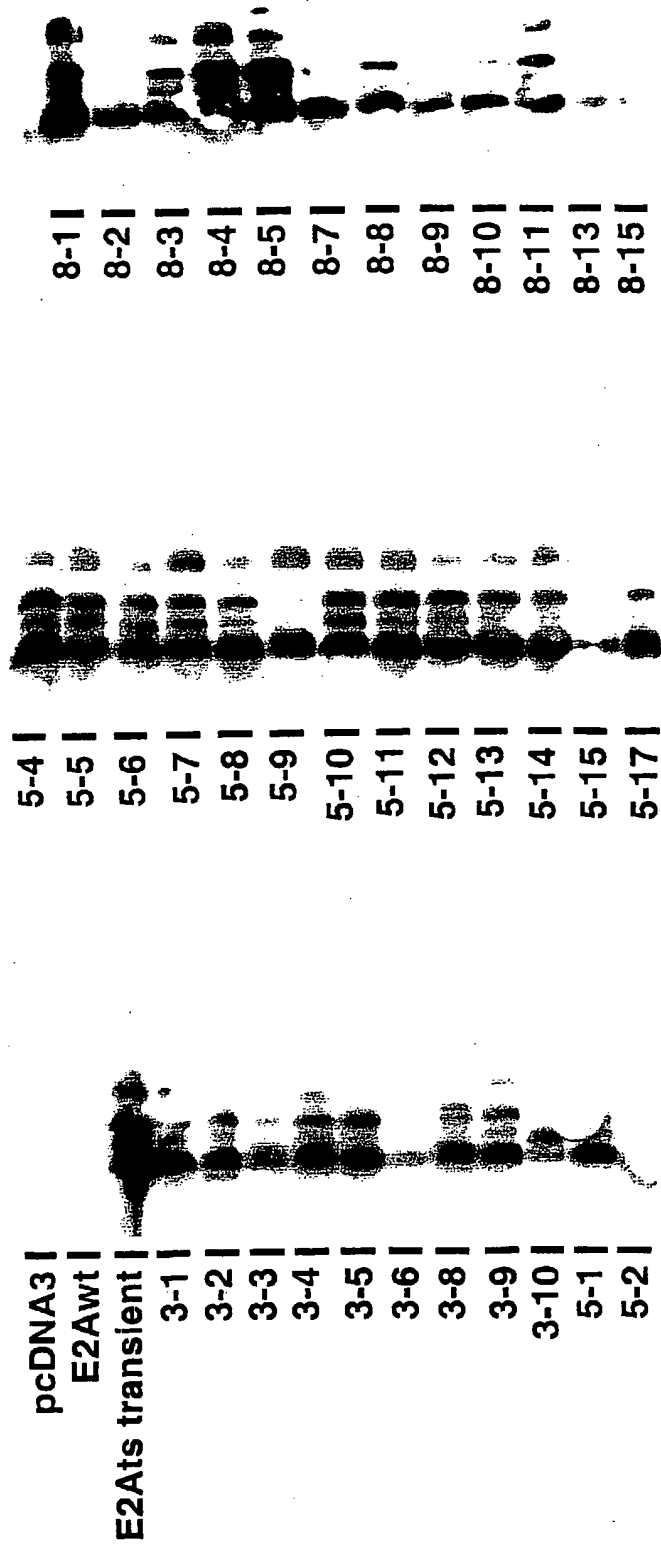
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**Figure 30 Temperature dependent growth of  
PER.C6**



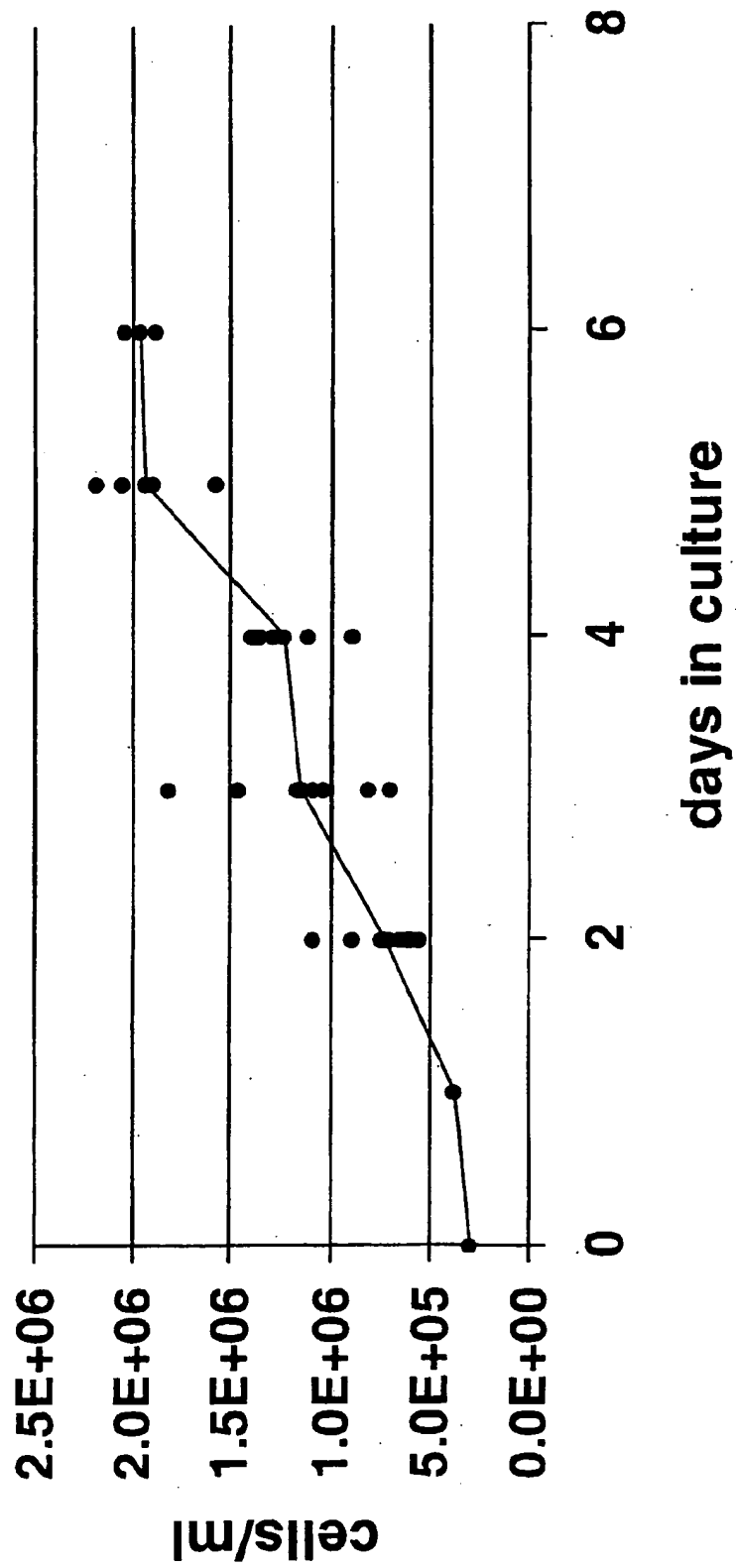
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**Figure 31** DBP levels in PER.C6 cells transfected with pcDNA3, pcDNA3wtE2A or pcDNA3ts125E2A

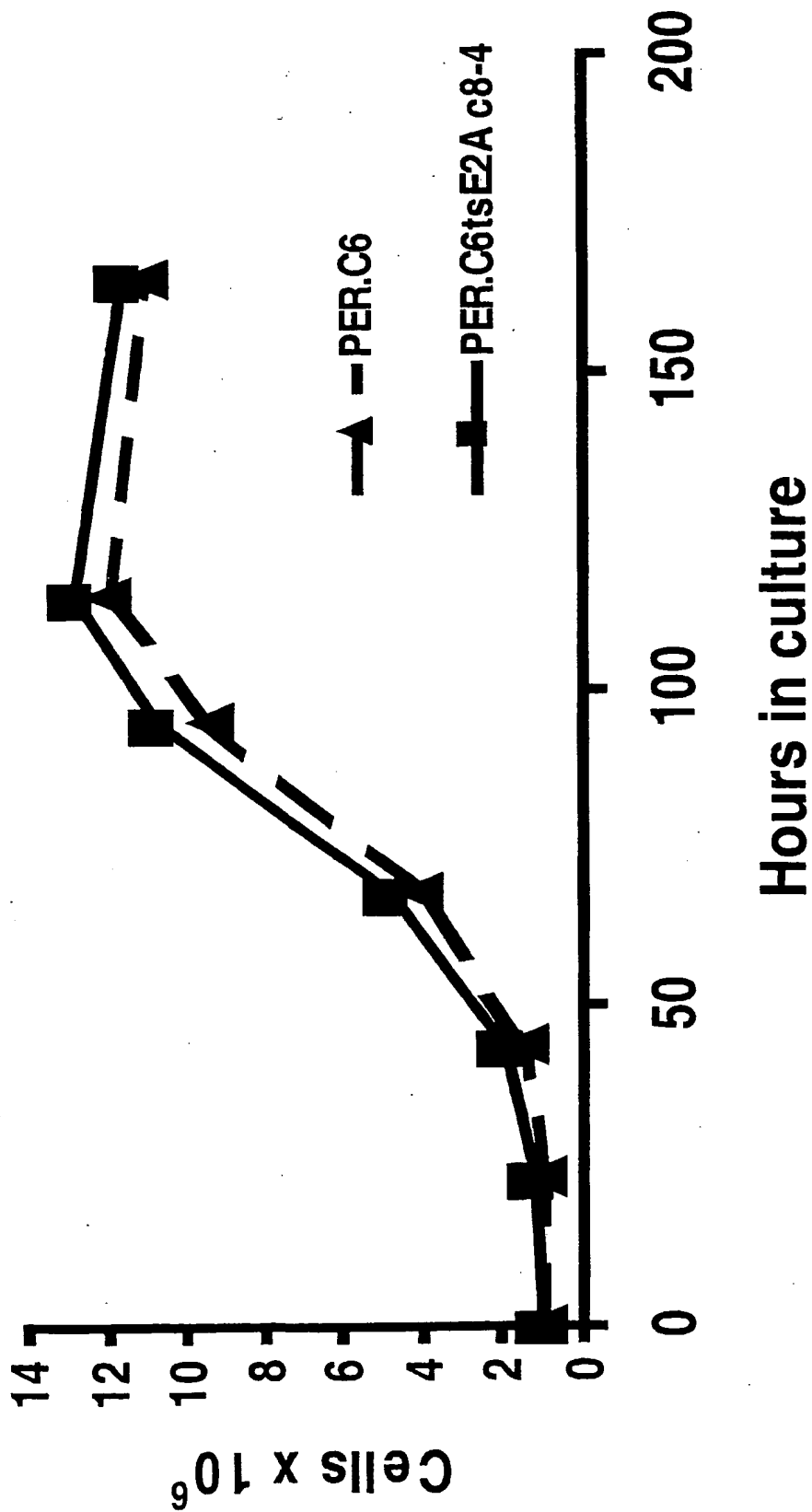




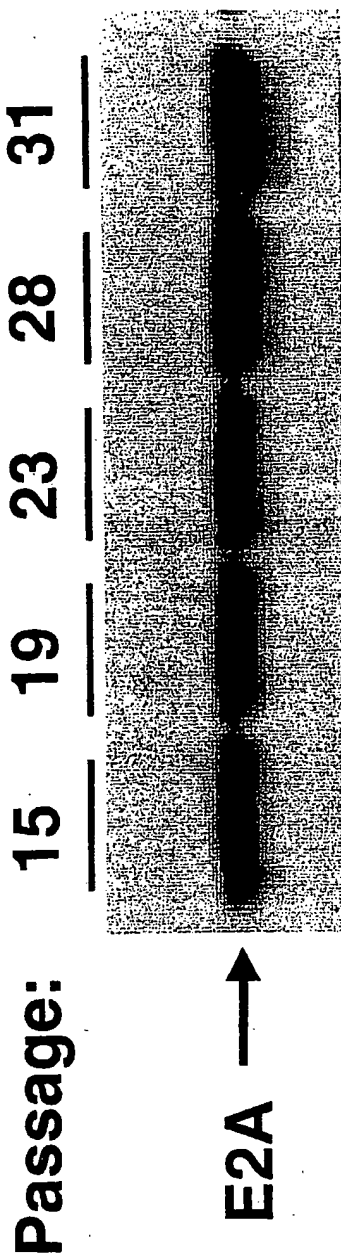
**Figure 32 Suspension growth of PER.C6ts125E2A C5-9**



**Figure 33** Growth curve PER.C6 and PER.C6tsE2A

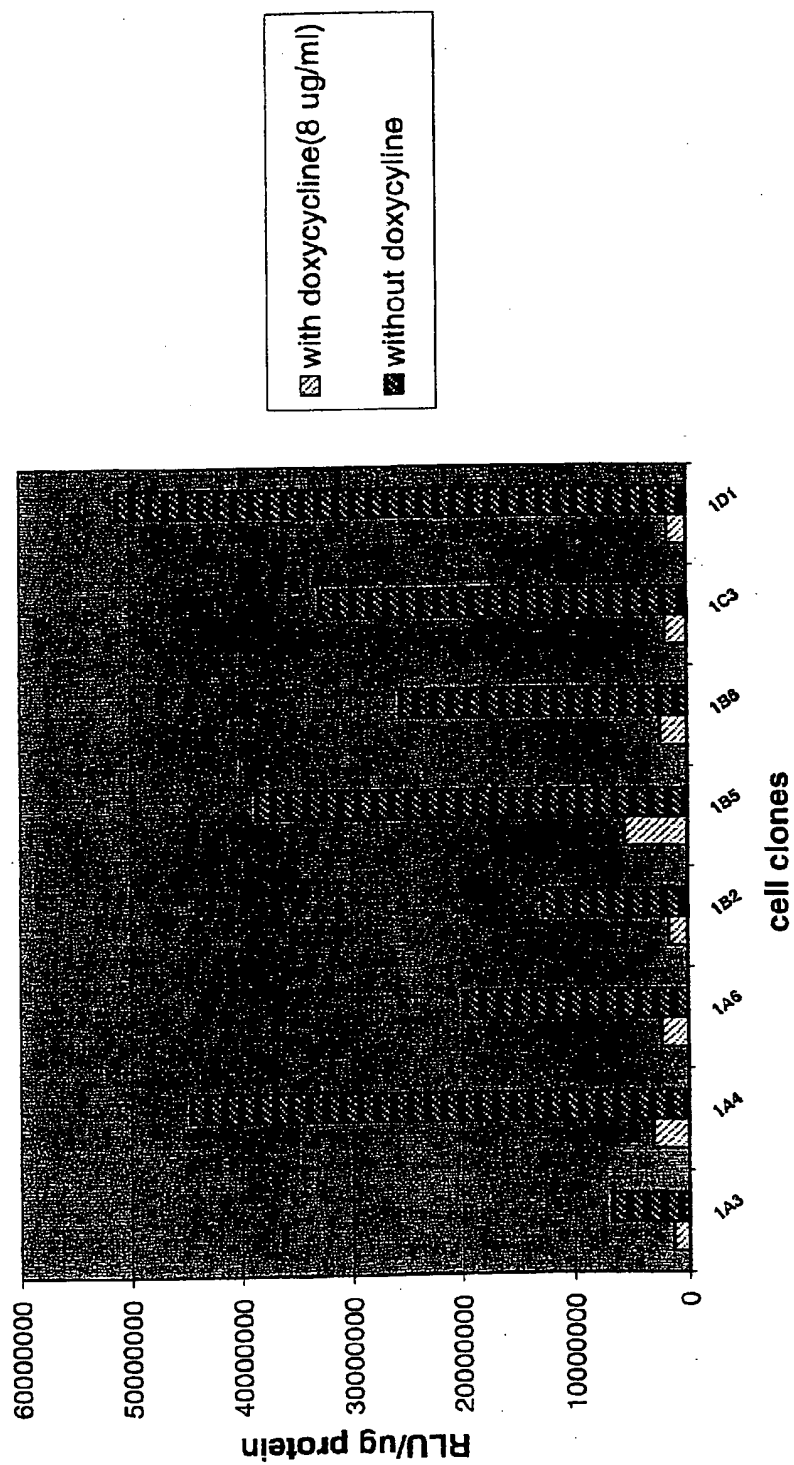


# Figure 34 Stability of PER.C6ts125E2A

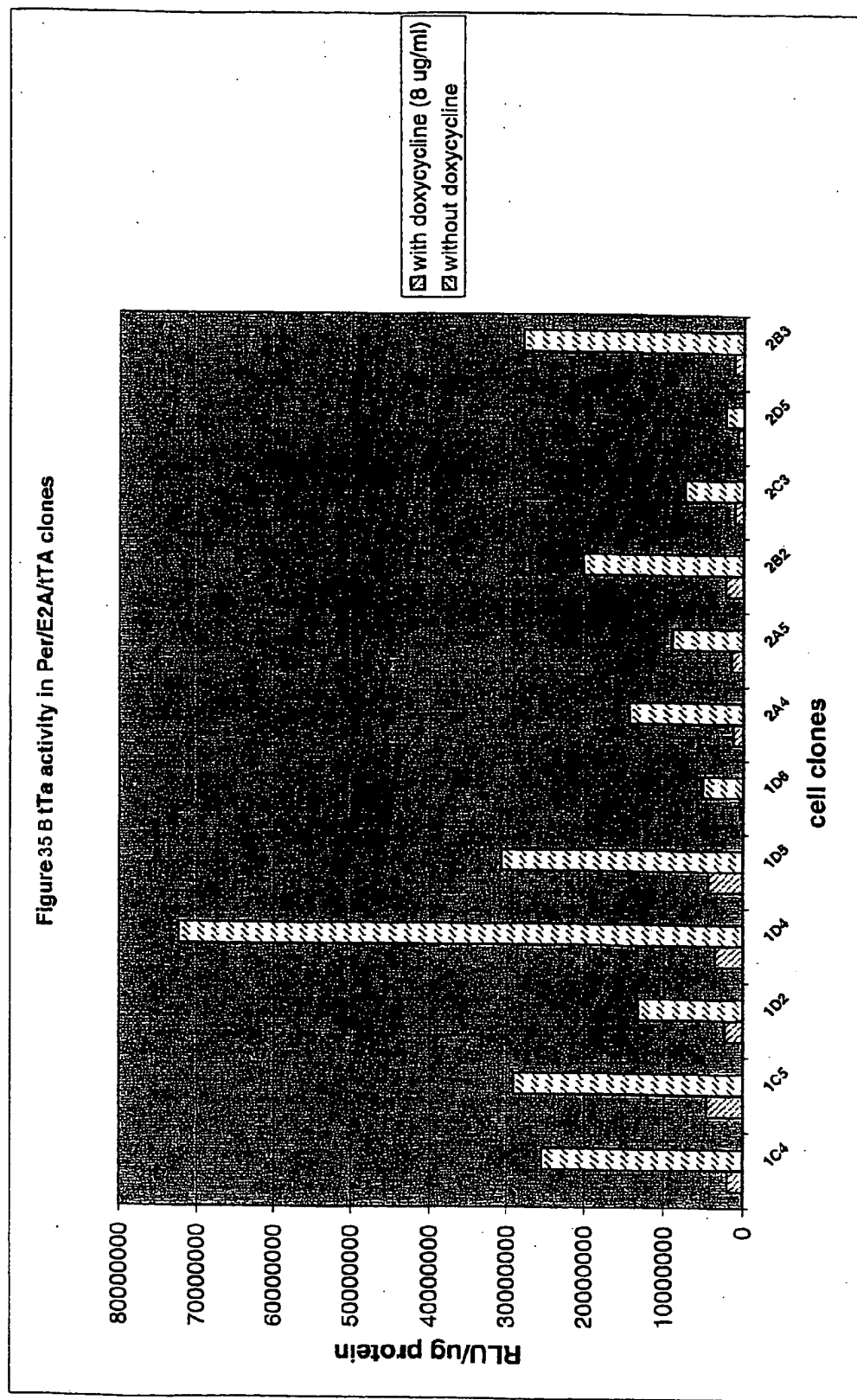


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Figure 35A tTA activity in Per.C6/tTA clones



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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/NL 00/00325

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/86 C12N15/34 C12N5/10 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EMBASE, CHEM ABS Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	cited in the application the whole document	1-3,5-7, 10-19, 21,22
X	WO 96 12030 A (RHONE-POULENC RORER SA ; LEE MARTIN (FR); PERRICAUDET MICHEL (FR)) 25 April 1996 (1996-04-25)	23
Y	the whole document	1-3,5-7, 10-19, 21,22
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A	WO 97 12986 A (CORNELL RES FOUNDATION INC) 10 April 1997 (1997-04-10) page 11, line 3 - line 35 page 13, line 1 - line 30 ---	1-23
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International Application No

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Information on patent family members

International Application No

PCT/NL 00/00325

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			JP 11514866 T	21-12-1999
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			EP 0946742 A	06-10-1999

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